Biochemical Studies on Ethanol and Denervation Induced Muscle Atrophy

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ABSTRACT

Enzymes activities belonging mainly to three systems were determined in muscles from two animal models of muscle atrophy. The first model is alcoholic myopathy. The other is surgical denervation. In the denervation model, the effect of a ß-agonist, clenbuterol, was also studied. Clenbuterol has been reported to ameliorate denervation induced atrophy. The three enzyme systems investigated were lysosomal enzymes including proteinases, free radical scavenging enzymes and energy metabolizing enzymes.

In alcoholic myopathy, activities of the lysosomal enzymes, cathepsin B and acid phosphatase, increased in soleus muscle only. Alkaline phosphatase activity was also found to be increased in soleus. These provide evidences that it is mainly the slow twitch fibers, the main components of soleus, which undergo atrophy after ethanol treatment. For free radical scavenging enzymes, soleus showed a pattern different from that of the other muscles. Unchanged activities were found in soleus while increased catalase and decreased superoxide dismutase activity were found in other muscles studied. The change in activities of these enzymes may serve as a protective mechanism for the alcohol induced damage. As for energy metabolism, soleus shifted from oxidative metabolism to anaerobic glycolysis.

After denervation, muscle weights decreased. Clenbuterol treatment restored the weight of muscles, with

the least response in EDL. Here, a fiber type specific effect was also found. Enzymes of energy metabolism, both in anaerobic glycolysis and in oxidative metabolism, were decreased after denervation. Clenbuterol reversed the change in MDH activity in all muscles studied and reversed the LDH activity mainly in slow twitch muscles. The effect of clenbuterol was specific to denervated muscles. The muscle type specific and denervation specific effect was also found in the activities of proteinases and free radical scavenging enzymes. In normal muscles treated with clenbuterol, only soleus under long term treatment showed a lowered proteinase activity. In denervated muscles, proteinase activities were increased. Clenbuterol had its ameliorative effect only in denervated fast twitch muscle. Lipid peroxide content in muscles of adult rats was not affected by denervation. It seems that lipid peroxidation may not be involved in the denervation induced damage in adult rats. However, superoxide dismutase activities increased only in denervated fast twitch muscles. This suggests that active radical species may be involved. This also proposed that denervation exerts its damaging effects in different types of muscle types through diverse mechanisms. For all muscles examined, a marked increase in catalase activity was found and this was almost completely restored by clenbuterol treatment.

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TABLE OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CK	creatine kinase
CP	creatine phosphate
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
EDL	extensor digitorum longus
EDTA	ethylenediaminetetraacetic acid
FeSOD	ferric superoxide dismutase
Glu-6-P	glucose-6-phosphate
GSH	reduced glutathione
GSH-peroxidase	glutathione peroxidase
GSSG	oxidized glutathione
GSSH-reductase	glutathione reductase
H_2O_2	hydrogen peroxide
Km	Michaelis constant
LDH	lactate dehydrogenase
MAD	malonaldehyde
MDH	malate dehydrogenase
MnSOD	mangano superoxide dismutase
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate

NADPH	reduced nicotinamide adenine dinucleotide phosphate
$O_2^{\cdot -}$	superoxide anion radical
OH·	hydroxyl radical
Pi	inorganic phosphate
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
SOD	superoxide dismutase
TA	tibialis anterior
TBA	thiobarbituric acid
Z-Arg-NNap	N^{α}-benzyloxycarbonyl-arginine-2-naphthylamine
ZnCuSOD	cuprozinc superoxide dismutase

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Muscle and Nerve

In animals, muscles have a large number of physiological functions. In vertebrate, there are three main classes of muscles that are characterized by both their appearance in the light microscope and their mode of innervation (Squire, 1986). They are the skeletal muscles, the smooth muscles and the cardiac muscles. Approximately 40 per cent of the body is made up of skeletal muscles and another 10 per cent is smooth and cardiac muscles (Guyton, 1987). Skeletal muscle comprises the greatest mass of somatic musculature. Contraction of skeletal muscle can be consciously controlled. For this reason, skeletal muscle is also classified as the voluntary muscle while smooth and cardiac muscles are referred to be involuntary muscles. During normal behavior, it is the interaction of peripheral nerves, the myoneuronal junctions and skeletal muscles with each other to bring about the contraction and relaxation of a muscle. It is therefore of paramount importance to understand the relationship between muscles and nerves.

1.1.1 Neuron and Nerve

Neuron is the basic building block of the nervous system. Its specialized function is to receive and transmit message in the form of neural impulses. A typical motor neuron has a few processes called dendrites which extend out from the cell body and arborize extensively. It has also a single, elongated structure of cytoplasmic extension called axon. Axon ends in a number of synaptic knobs. These knobs

contain granules or vesicles in which the synaptic transmitter secreted by the nerve is stored (Ganong, 1981). Dendrite and axon differ in their function by that dendrite accepts the signals from other neurons while axon conducts impulse away from the dendrite zone. Probably because it is so long, an axon is often referred to as a nerve fiber.

In essentially all cells of the body, electrical potentials exist across the membrane. This is called the resting potential. In a neuron, as well as in a muscle cell, it amounts to about -40mV to -90mV (Guyton, 1987). It is the result of a slight excess of positive ions outside the cell membrane and a slight excess of negative ions inside the membrane. Although most cells have a electrical potential across the cell membrane, only some are "excitable" — that is, capable of self-generation of electrochemical impulses at their membranes and, in some instances, employment of these impulses to transmit signal along the membranes. The most well-known excitable cells are the neurons and muscle cells. An electrical, chemical, or mechanical stimulus may alter resting potential of neuron by increasing the permeability of the membrane to sodium and cause depolarization. If the neuron membrane is only slightly stimulated, only a local disturbance may occur in the membrane. However, if the stimulus is sufficiently strong it may result in an action potential — that is, the propagation of a neural impulse. Actually, a neural impulse is transmitted as a wave of depolarization that travels down

the neuron. For neuron, the impulse is usually transmitted along the membrane from dendrite through the cell body to the axon at which chemical substance or electrical stimulus will be release and stimulate the following cells. In muscle cells, the signal will at last be converted into the contraction of the cells.

1.1.2 Skeletal Muscle

Muscle cells, like neurons, can be excited to produce an action potential that is transmitted along their cell membrane. They contain contractile proteins and, unlike neurons, they have a mechanism that can be activated by the action potential.

Skeletal muscle is made up of individual muscle fibers which are the "building blocks" of the muscular system. Most skeletal muscles begin and end in tendons, and the muscle fibers are arranged in parallel between the tendious ends so that the force of contraction of the units is additive. Each muscle fiber is a single cell, multinucleated, long, and cylindrical in shape.

The muscle fibers are made up of fibrils, and the fibrils are divisible into individual filaments. The filaments are made up of the contractile proteins (Figure 1. 1). For the special array of filaments in the fibrils, cross-striations can be observed on the muscle fibers.

Muscle contains the proteins myosin, actin, tropomyosin and troponin. Troponin is made up of 3 subunits, troponin I, troponin T, and troponin C. The arrangement of the

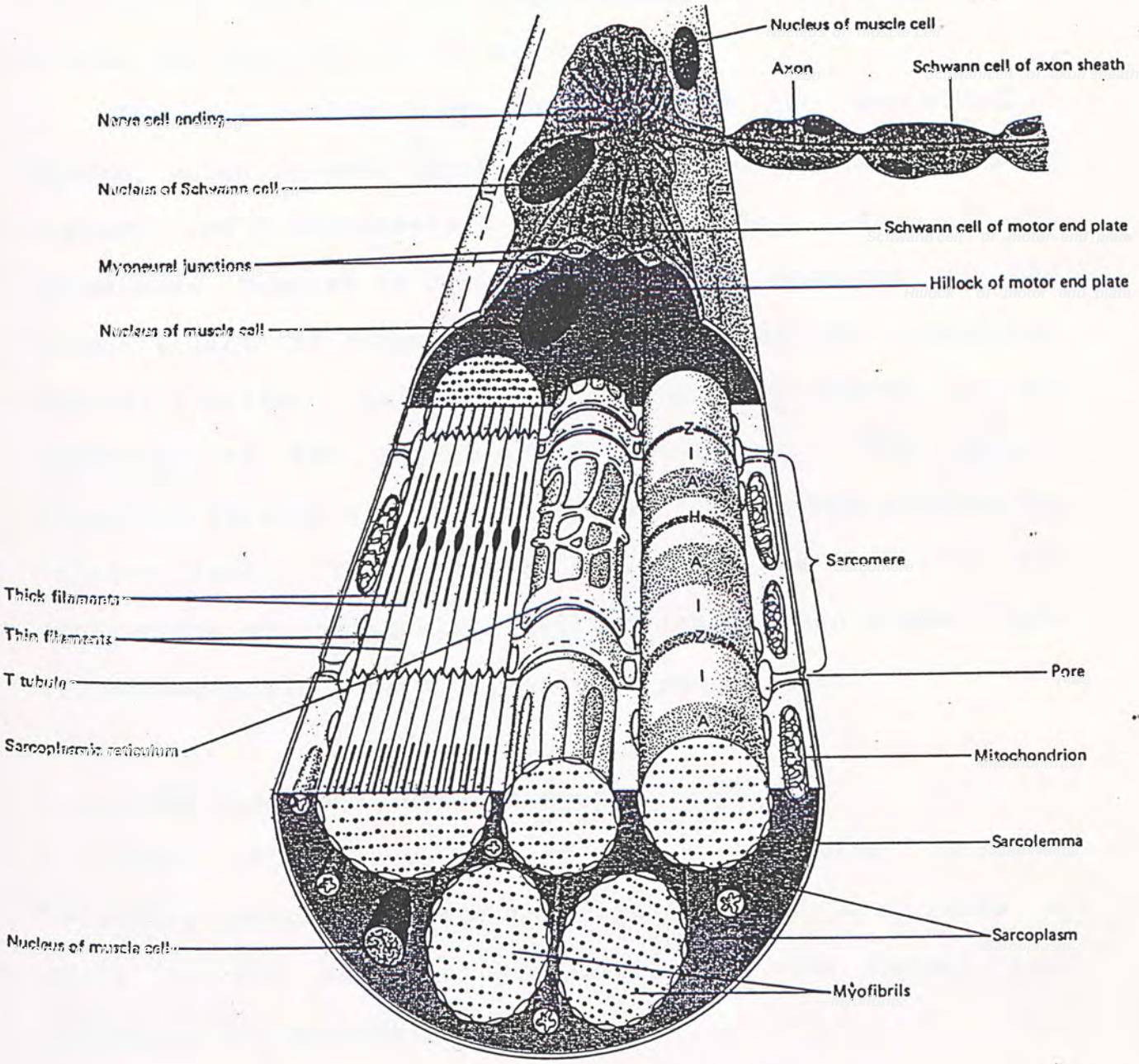

Figure 1.1 The structure of a skeletal muscle fiber (From Solomon, E.P. and Davis, P.W. : "Human anatomy and physiology" Saunders College Publishing : 191-210, 1983.

contractile proteins is shown in Figure 1.2. It is suggested that M-line and Z-line organize the array of contractile proteins and the degradation of these lines causes the disorder of the filament.

The muscle fibers are surrounded by the sarcotubular system, which is made up of the transverse tubular system (T system) and a sarcoplasmic reticulum. The T system of the transverse tubules is continuous with the membrane of the muscle fibers and forms a grid perforated by the individual muscle fibrils. Calcium ions are normally stored in the cisterna of the sarcoplasmic reticulum. The action potential spreads via the transverse tubules and release the calcium ions. The released calcium ions initiate the contraction of the muscle. Calcium ion is then pumped into the sarcoplasmic reticulum, and the muscle relax.

1.1.3 The Functional Nervous System

The motor nerve fibers which innervate striated voluntary muscles except those in the head are axons of cells in the anterior grey matter of the spinal cord (Goodgold and Eberstein, 1972).

The functional junction of nerve and muscle cell is called the myoneural junction or neuromuscular junction. It is also referred to be the motor end-plate. It is located at the midpoint of the fiber.

When a nerve impulse reaches the ends of the nerve cell, it induces the release of a transmitter substance. In voluntary muscle-nerve junctions this transmitter is

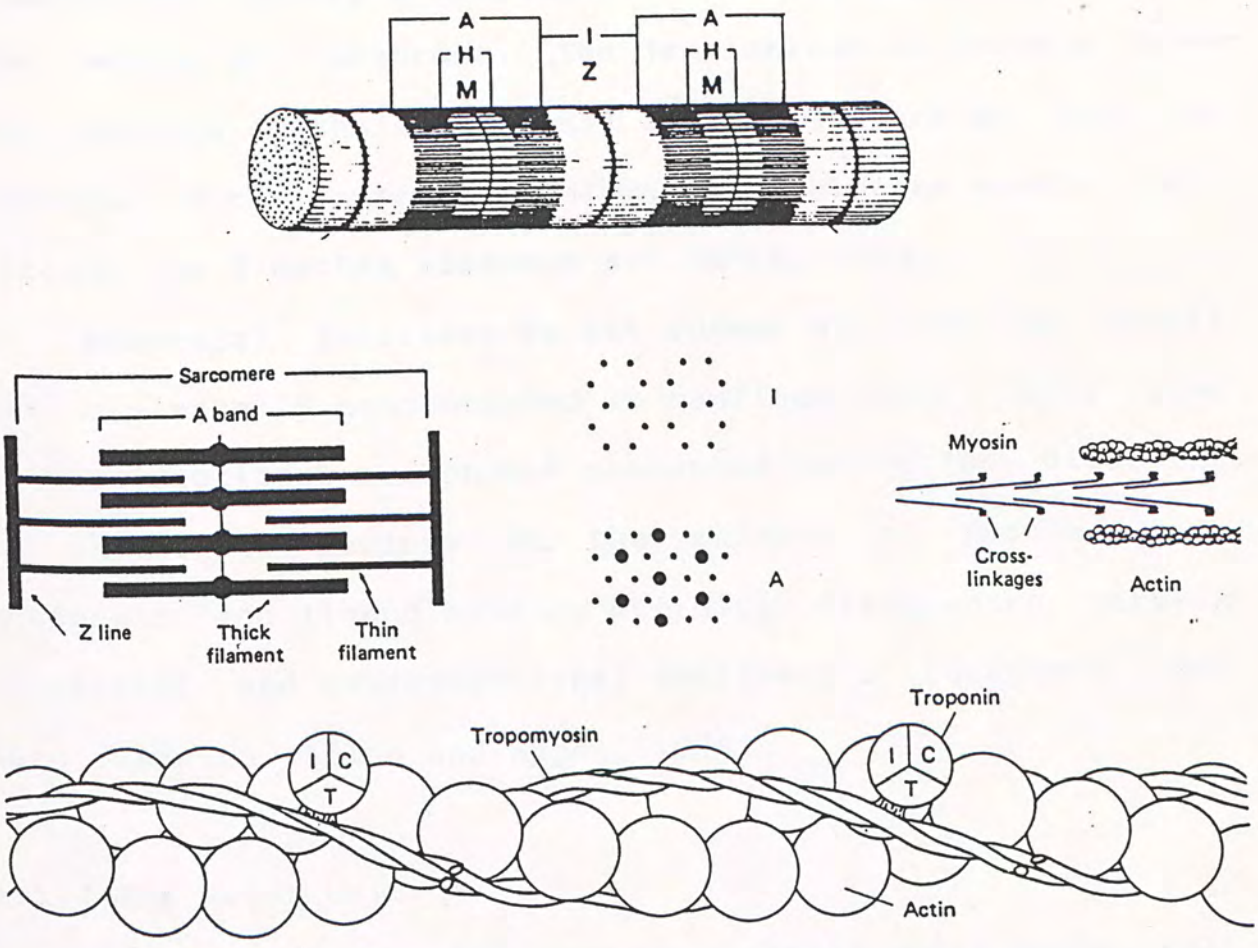

Figure 1.2 Top: The band pattern of a myofibril. Middle left: Arrangement of thin (actin) and thick (myosin) filaments in skeletal muscle. I and A represent a cross section through the I band and the lateral portion of the A band, respectively. Middle right: Detail of structure of myosin and actin. Bottom: Diagrammatic representation of the arrangement of actin, tropomyosin, and the 3 subunits of troponin (I, C, and T). (From Bowman, W.C. and Rand, M.J.: "Textbook of pharmacology" 2nd edition, Balckwell Scientific Publications, 1980 and Ganong, W.F.: "Review of Medical Physiology" 10th edition, Maruzen Asian Edition, Lange Medical Publications, 1981)

acetylcholine. It rapidly diffuses across the myoneural cleft, and when it reaches the muscle cell it almost immediately induces a temporary electrical depolarization of the muscle cell membrane. The depolarization travels down the surface of the muscle cell like a wave and at last be conveyed to the contractile elements within the muscle cell through the T system (Solomon and Davis, 1983).

Myoneural junctions do not spread all over the muscle but are usually concentrated in confined zones. This zone is the junctional region and possesses properties different to the other regions of the surface of muscle cell. Antigenic and ligand binding affinity differences between junctional and extrajunctional cholinergic receptors have been reported (Almon and Appel, 1976).

1.1.4 The Motor Unit

Within a muscle, the axon of a single motor nerve cell arborizes into many terminal branches. Each branch is attached to an individual muscle fiber. The branching of the axon permits a single neuron to stimulate a group of muscle fibers. The functional unit of the neuromuscular system is the motor unit; it consists of the anterior horn cell, its axon and all the muscle fibers innervated by that axon. It was indicated that the fibers which belong to a given motor unit are homogenous histochemically. Although there may be considerable intermingling of fibers of different units in a muscle, it appears that there is no

overlapping of fiber types within the single motor unit.

1.2 Structural Refinements in Vertebrate Muscles : Fiber Types

1.2.1 Different Types of Muscle Fibers

1.2.1.1 Tonic and Twitch Muscles

Vertebrate skeletal muscle can be divided into two main types, tonic and twitch (or phasic, tetanic) types, depending on their mode of innervation and response (Squire, 1986; Squire, 1981). Tonic muscles are multiterminally innervated, whereas twitch muscles are stimulated by a single motor nerve ending. The twitch muscle, like the nerve membrane, shows an all-or-none response, while the tonic muscle gives response graded according to the frequency of stimulus of the nerve.

Electron microscopy has shown that the most important structural difference between the two types of fibers relates to their transverse tubular systems and sarcoplasmic reticula (Bowman, 1980). Tonic muscles respond to excitation with a slow and maintained shortening or tension increase, in contrast to the rapid and brief contractile response of twitch muscle fiber. The different arrangements and numbers of contacts between t-tubules and sarcoplasmic reticulum in the two types of fibers are compatible with their different contractile velocities.

In mammals the twitch muscles predominate. Tonic muscles occurs principally in the head and neck region: in the eye, the voice box, and the ear. Almost all other

skeletal muscles are twitch muscles. In this thesis, the focus is on the twitch muscles.

1.2.1.2 Fast Twitch and Slow Twitch Fibers

Although almost all of the muscles in mammals are twitch muscles, there is still a considerable range of contractile response given by the skeletal muscles. One of the main reasons is that different muscles contain varying proportions of individual fiber types that have distinct properties. The twitch fibers can be classified into three categories on the basis of three criteria, (1) the contraction time of the fiber relative to others within the same animal, (2) glycolytic capacity, and (3) oxidative capacity (Peter et al., 1972). They are the type I (also is called slow twitch red intermediate or slow oxidative) fiber, the type IIb (also called fast twitch pale, or fast glycolytic) fiber, and the type IIa (also called fast twitch red, or fast oxidative glycolytic) fiber. Both the type I and type IIa fibers have a high myoglobin content which accounts for their red color. On the other hand, type IIa and type IIb fibers have rapid rate of contraction and relaxation, whereas type I fibers are relatively slow in contracting and relaxing.

The muscle types depend primarily on two different forms of energy supply. Slow muscles (type I) depend largely on ATP synthesis associated with mitochondria and an ample oxygen supply (the oxidative system), whereas fast

muscles (type IIa and IIb) depend largely on glycogen and anaerobic phosphorylation (the glycolytic system). Thus, the slow fibers have an abundance of mitochondria, relatively little glycogen, a poorly developed sarcoplasmic reticulum and the presence of the oxygen carrier myoglobin. Fast fibers have fewer mitochondria, an abundance of glycogen and a very well-developed sarcoplasmic reticulum.

The fiber types can be distinguished qualitatively by histochemical staining for myofibrillar actinomyocin ATPase, or for enzymes of distinct metabolic functions, such as anaerobic carbohydrate catabolism or aerobic substrate end oxidation (Pette et al., 1979). By analyzing 10 different enzymes in individual fibers from human biceps, two clearly defined fiber types and several subtypes were distinguished (Lowry et al., 1978). They also demonstrated that the presence among neighboring muscle fibers of a broad spectrum of highly coordinated enzyme activity.

On the basis of histochemical tests on different enzyme systems, a fourth category of fiber was also reported (Brook and Kaiser, 1970; Squire, 1986). It is also fast twitch and is termed type IIc. This type of fiber normally present in only small numbers in adult human muscles, but they can be more abundant in young muscle or in pathological cases.

On the other hand, the patterns of myofibrillar proteins obtained in capillary electrophoresis can be classified into two types which were correlated to the two different fiber types (Pette et al., 1979). This illustrated that the ultrastructural differences between

different histochemically defined fiber types. Comparison of myofibrillar proteins from fast and slow-twitch skeletal muscle showed differences in myosin light chains, tropomyosin, the inhibitory and tropomyosin-binding components of troponin, (troponin I and troponin T, respectively) and α-actinin (John, 1976). Using electron microscopy it was found that characteristic ultrastructural variations occur in the M-bands and Z-bands of different fibers and in other regions, such as myosin heavy and light chain and of many other proteins (Squire, 1986). This may form the basis that different types of fibers have different vulnerability to the damage caused by chemical or other treatments (Haller, 1985).

The speed of contraction of a muscle as a whole depends on the proportions of the fiber types of which it is composed. Type IIb fibers are adapted for short term powerful plastic activity and predominate in flexor muscles, such as the extensor digitorum longus, and the gastrocnemius. Type IIa fibers are adapted for sustained plastic activity and predominate in muscle such as the diaphragm. Type I fibers are adapted for economical, low speed, sustained tonic activity. They predominate in deep extensor muscles muscles, such as the soleus and the cureus, that are mainly involved in the maintenance of posture (Bowman, 1980).

For the large difference in many aspects, the different types of muscle fibers may have quite different response to

the disease state.

1.2.2 Formation and Maintenance of Different Types of Fibers

In the early stages of development, all limbs muscles are slow-contracting and differentiation into fast or slow types occurs only in a few weeks. The oxidative system has been developed in muscles at the late embryonic stage. The postnatal fiber development produces much more growth in some fibers than the others, the larger fibers tend to become less dependent on oxidative enzymes and better supplied with glycogen and the glycolytic enzymes (type II fibers). The smaller fibers (type I fibers) remain largely oxidative (Squire, 1986). This is the origin of different types of muscle fibers.

Because the development of early muscle fibers into various well-defined fiber types occurs at a time when motor nerves are active, it was thought that fiber type differentiation was due to the nervous input. This idea was indicated by cross-innervation experiments. Buller et al. (1960) showed that when motor nerves of a fast twitch muscle and of a slow twitch muscle were cut, exchanged, and then allowed to re-innervate their new host muscles, the slow muscle would become faster and the fast muscle would become slower. Clearly the type of motor nerve serving a muscle controls in some way the nature of the contractile machinery.

The nature of the control of motor nerve on the muscle fiber differentiation was found by Salmons and Sreter

(1976). They showed that the important factor is not difference in the nerves (a trophic factor) but rather the pattern of electrical activity they carry. Also, it was found that in the transformed fibers, the fiber type specific characteristics such as protein polymorphic patterns, M-band structure, etc. changes as the physiological property changes (Squire, 1986). This suggests that the impulse activity of a motor nerve somehow modulate gene expression, so that after cross-innervation some genes are suppressed and others are turned on. However, cross-innervation did not affect the proportion of the polymorphic forms of tropomyosin, implying that nerve-mediated factors are not the only factors involved in regulating the genes for the proteins of the myofibril (John, 1976).

On the whole, the action of motor nerves appears to involve in the initiation and maintenance of the differentiation of muscle fibers.

1.3 Trophic Interactions Between Nerves and Muscles

When skeletal muscle is deprived of its nerve supply, it gradually undergoes a series of physiological, biochemical, and morphological changes, which culminate in atrophy. It is clear that virtually all nerve cells are involved in similar long-term relationships with other cells. This is referred as the neurotrophic phenomena. The definition of neurotrophic phenomena is the long-term

relationships in which nerve cells and target cells interact so as to influence the structure or function of either member of the pair (Drachman, 1976).

The neurotrophic phenomena in skeletal muscle can be roughly classified into three broad categories.

(a) Maintenance of the integrity of muscle. Denervation cause various changes and atrophy in muscles while administration of soluble extract of peripheral nerve significantly ameliorated the atrophy (Davis et al., 1988). The ability of the motor nerve to prevent the denervation changes is an important aspect of its trophic functions.

(b) Regulation. Regulation of certain metabolic and physiological properties of muscles takes place in a way that would adapt them for their appropriate tasks, such as the initiation and maintenance of the differentiation of muscle fibers as discussed above.

(c) Formation and maintenance of neuromuscular junctions. In embryonic development, in cell culture, and in regeneration, nerve forms connections with skeletal muscle. Both the initial formation and the subsequent maintenance of neuromuscular junctions are presumably under neurotrophic influence (Drachman, 1976; Ishikawa et al., 1988).

Although the range of neurotrophic effects is large, the number of possible ways by which the motor nerve can theoretically transmit trophic instructions to skeletal muscle is limited. The only possible ways are cholinergic

transmission, release of non-cholinergic trophic substances, and nerve-muscle interaction.

By using various methods, including neurotoxins administration to block the activities of the nerve and disuse, it was found that the trophic control of skeletal muscle is mediated by more than one kind of neural influence (Drachman, 1976). Cholinergic transmission plays an important role in the regulation of many, but not all, properties of skeletal muscle. Muscle usage is also important in maintenance of the muscle normality but it cannot constitute the whole effect of cholinergic transmission, particularly in the type I fibers (Nemeth et al., 1980). It is suggested that neurogenic trophic substances also exert an effect on one or more of the highly specialized morphologic, biochemical, or physiological properties of muscle (Davis et al., 1988). However, the nature and mechanism of these non-cholinergic trophic substances are not yet known. The mechanism of the trophic effect of motor nerves on muscles is still largely unknown.

1.4 Energy Metabolism of Skeletal Muscle

1.4.1 Energy Sources of Skeletal Muscle

Muscles convert stored chemical energy into mechanical energy. The immediate source of energy is the energy-rich organic phosphate derivatives in muscle while the ultimate source is the intermediary metabolism of carbohydrates and lipids.

The hydrolysis of ATP provide directly the energy for muscle contraction. But there also exists in muscle another energy-rich phosphate compound, phosphocreatine (CP), that can supply this energy. When CP is hydrolyzed to creatine and phosphoric acid, energy will be released. The energy of ATP is stored in the form of CP by transferring its phosphate group, together with much of the energy contained in its chemical bond, to creatine. When needed, CP transfers its energy back to ADP, forming ATP, which is then used to provide the energy needed for muscular contraction. It is believed that this somewhat round about system of energy transfer renders the muscle cell more resistant to oxygen deprivation then would be the case if ATP alone were employed (Solomon and Davis, 1983). Moreover, since CP is used only to power contraction, other cellular processes cannot compete directly with contraction for the energy of CP.

Fatty acids are by far the most important fuel of resting and sustained exercising muscles, implying aerobic metabolism in which substrate oxidation by mitochondria is paramount. During exercise the glycogen store serves as fuel for glycolysis. Thus the muscle possess large emergency stores of food in the form of glycogen. As exercise continues, an increasing amount of blood glucose is taken up. Type IIb fibers play little part in resting muscle metabolism, but are increasing recruited as the demand for power increases. Presumably the type IIa fibers also play a greater role in sustained exercise (Ellis,

1978). Since type IIb fibers process only anaerobic glycolysis, the end waste product is lactic acid, the fast twitch muscle has a large amount of lactate dehydrogenase (LDH) which enables the continuous running of anaerobic glycolysis.

1.4.2 Energy Metabolism in Myopathy States

Energy metabolizing enzyme activities were found to be altered in various types of myopathies. Such change in activities may serve as an indicator of the pathological damage to the muscle.

In dystrophic mouse gastrocnemius most of the NADP-requiring dehydrogenases increase while NAD-requiring enzymes decrease or unchanged. The most marked increases were found in the pentose shunt enzymes, glucose-6-P dehydrogenase and 6-P-gluconic dehydrogenase. The most marked decreases were found in the glycolytic enzymes, glycerol phosphate dehydrogenase and LDH. The activities of individual enzyme suggested the overall decrease in glycolysis. Regarding carbohydrate metabolism, the pattern of enzyme activity changes suggested a shift from normal glycolytic and oxidative pathways to the pentose phosphate shunt (McCaman, 1963). This suggested that selective loss of the more highly-specialized type II fibers was occurring. However, there are two exceptions to this simple interpretation, namely, the increased activities of hexokinase and isocitrate dehydrogenase. On the other hand,

many mitochondrial enzyme activities remained at normal levels until the disease is well advanced (McCaman, 1963). Also, the change of metabolic substrate levels suggested increased lipogenesis via extra-mitochondrial citrate cleavage and esterification of glycerol phosphate (Ellis, 1978).

By the change in LDH and creatine kinase isozyme patterns, it was postulated that muscular dystrophy disease in general conferred a metabolic pattern in which "mature" forms of enzymes disappeared and "immature" forms replaced them (Reichmann and Pette, 1984; Ellis, 1978). Both the shift in metabolism and the pattern of isozymes reflected the occurrence of "de-differentiation". However, distinct differences concerning mainly the mitochondrial enzymes and hexose kinase in immature and dystrophic muscles had been established and thus implying that dystrophic muscle does not simply fail to mature but rather that its development is overlain by the malignant effects of disease (Ellis, 1978).

In denervated muscles, enzymes involved in anaerobic glycolysis, citric acid cycle, and ß-oxidation of fatty acids showed increase activities in both types of muscles (Simard, 1985). The marked reduction in anaerobic activities in type II fibers (Shackelford and Lebherz, 1981) and the increase of fetal type isozyme of CK in denervated muscle (Matsushita et al., 1987) suggested that denervation results in a partial "de-differentiation" of muscle fibers and the trophic effect of nerve on muscle differentiation. Also, increase activities of hexokinase as found in

dystrophy was also reported in slow muscles (Simard, 1985).

By the similarity of the energy metabolizing enzyme pattern in denervated and dystrophic muscles, it seems that the enzyme changes may represent similar secondary effects caused by different agents (McCaman, 1963) or the involvement of motor nerve in dystrophy. However, the nature of these changes in the myopathies is still not clear.

1.5 Protein Turnover and Proteinases in Skeletal Muscle

1.5.1 Protein Turnover

Most cellular proteins are in a dynamic state of constant turnover. Here, "protein turnover" or "protein degradation" refers to the hydrolysis of intracellular proteins to their component amino acids (Goldberg and St.John, 1976). This process is involved in basic cellular functions, since the levels of intracellular proteins are determined both by the rates of synthesis and the rates of degradation. An additional role of protein breakdown appears to be the provision of amino acids in times of need, since overall rates of protein degradation are greatly accelerated under conditions of nutritional or hormonal deprivation. Another important function is the disposal of defective proteins : abnormal proteins produced by specific mutations or by various experimental manipulations are selectively recognized and rapidly degraded (Goldberg and Dice, 1974; Hershko and Ciechanover, 1982).

The proteins of muscle, in common with other tissues, undergo constant degradation and replacement (Goldberg, 1969a; Pennington, 1977). Changes in the rate of protein breakdown may contribute to normal muscle growth in the work-induced hypertrophy (Goldberg, 1969a) and the atrophy resulting from denervation (Goldberg, 1969b; Goldspink, 1976), dystrophy (Katunuma and Noda, 1982), vitamin E-deficiency (Dayton et al., 1979) and cortisone treatment (Goldberg, 1969b). We know a lot about the factors influencing protein degradation but a little about their mechanisms (Libby and Goldberg, 1980). The biochemical basis for the failure to regulate protein catabolism in these pathological states is not known. Further studies in this area may clarify the intracellular mechanism for protein degradation, and may even lead to improved therapeutic procedures (Goldberg et al., 1982).

Muscle contains many proteinases, both lysosomal and non-lysosomal, that could degrade cell protein in vivo (Pennington, 1977). However, in muscles the role of any given proteinase or organelle in the breakdown of average cell protein is unknown (Libby and Goldberg, 1980).

1.5.2 Proteolytic System

The systems or organelles of the cell that are responsible for the degradation of cellular proteins must contain endoproteolytic and exoproteolytic activities but may also contain other enzymatic activities to achieve the

end point, namely complete hydrolysis. An obvious locus for degradative process is the lysosome with its high concentration of hydrolases (including proteinases, usually referred to as cathepsins). However, the nonlysosomal proteinases are also responsible for degradation of cellular proteins (Beynon and Bond, 1986).

Proteolytic enzymes are classified mechanistically according to the functional residues at their active sites: serine, cysteine, aspartic, or metalloproteinase (Barrett, 1977). In general, the lysosomal proteinases are of the cysteine and aspartic class, cytosolic proteinases are of the cysteine class and membrane-bound proteinases are often metallo- or serine proteinases. Proteinases that act extracellularly are generally serine proteinases (trysin, blood proteinase), but metalloproteinases (collagenases) and aspartic proteinases (pepsin) also function outside of cells (Beynon and Bond, 1986).

1.5.2.1 Lysosomal Proteinase

The lysosomal system is well suited for a major role in endogenous proteolysis because it is able to sequester cellular material by the process of autophagy and has immense proteolytic capacity (Dean and Barrett, 1976). Lysosomes of most tissues contain at least four well-defined endopeptidase (cathepsin B, cathepsin D, cathepsin H and cathepsin L), one exopeptidase (cathepsin C, a dipeptidyl aminopeptidase), and several peptidase (e.g. cathepsin A). Also, a process of autophagic engulfment of cellular

constitutes has been observed under a variety of pathological or physiological conditions (Hershko and Ciechanover, 1982).It is generally accepted that lysosomes are responsible for hydrolyzing pinocytized and phagocytized proteins (Goldberg and St.John, 1976; Katunuma and Noda, 1982).

Role of lysosomes in protein degradation has been investigated extensively. Degradation of most short-lived proteins or abnormal proteins is probably nonlysosomal in nature. (Neff et al., 1979). For degradation of long-lived proteins, most or all of enhanced protein breakdown in mammalian cells is carried out by the lysosomal system, but there are conflicting reports concerning the involvement of the lysosomal pathway in the basal degradation of these proteins. It is possible that the proportion of lysosomal pathway is greater in non-growing tissues, such as the normal liver or muscle (Hershko and Ciechanover, 1982) and in cells where proteolysis is accelerated (Neff et al., 1979).

It has been suggested that lysosomal proteinases contribute to the degradation of myofibrillar proteins. Cathepsins B, H and L are thiol proteinases and cathepsin D is a aspartic proteinase in lysosome. They all have optimum activities at acidic environment. The degradation of purified myofibrillar proteins by cathepsins B and D was clearly demonstrated by sodium dodecyl sulfate/poly acrylamide-gel electrophoresis by Schwartz and Bird (1977).

The ability of cathepsins to degrade various myofibrillar components is also investigated. Cathepsin B can degrade some myofibrillar proteins, such as myosin, troponin and tropomyosin. Cathepsin H cannot degrade any native myofibrillar proteins except troponin T (Katunuma and Noda, 1982). Also, it was found that cathepsin D degraded the Z-line and filaments adjacent to the H-zone some myofibrillar proteins, such as myosin, troponin, tropomyosin. Cathepsin L causes the fragmentation of myofibrils, and degraded the Z-line and M-line (Matsukura et al., 1984). Cathepsin L is the most important endopeptidase among the lysosomal thiol proteinases for its high proteolytic activity and its ability to disassemble the myofibrils.

The exact role of lysosomal proteinase in muscle is not clear. Cathepsin activities were found to be elevated in muscle wasting conditions, such as denervation (Maskrey et al., 1977), toxin-induced muscle paralysis (Tågerud et al., 1986), and Duchenne muscular dystrophy (DMD) (Katunuma and Noda, 1982). Also, enhanced protein breakdown was shown in muscles lacking in nutrients and in insulin through a lysosomal process (Furuno and Goldberg, 1986). Cathepsin L was also supposed to be responsible in the postmorten aging of muscle (Matsukura et al., 1984). Thus, the role of lysosomal proteinases in wasting muscles is rather confirmed. On the other hand, it has been reported that the reaction products of cathepsin B and D was found in two "types" of lysosomes in muscle cells (Bird et al., 1980). This shows the involvement of these enzymes in the

protein degradation of muscle cells. However, Libby and Goldberg (1981) suggested that although the lysosome is the primary site for the acetylcholine receptor degradation and perhaps for degradation of other surface proteins, the breakdown of most proteins in myotubes seems to involve a distinct proteolytic system requiring metabolic energy. So, the role of lysosome in protein degradation in normal cell is not clear.

1.5.2.2 Calpains and Other Cytosolic Proteinases

Cytosolic proteinases have been isolated and characterized from many cell types in the last 10 years. It is now clear that significant proteolysis takes place in the cytosolic compartment of cells and that this is important in the activation, posttranslational processing, and initiation of extensive degradation of proteins (Beynon and Bond, 1986). The proteinases that have been isolated from the cytosol of various cells are cysteine proteinases, and many have requirements for activation ligands, e.g., Ca^{2+} for the calpains, ATP for the ATP-dependent proteinases (Beynon and Bond, 1986).

Proteinases having a neutral pH optimum and an absolute requirement for calcium ions are found in virtually all mammalian cells (Mellgren, 1987). They are known as calpains (CALcium-dependent paPAIN-like proteinases). They are also known as calcium-activated neutral proteases (CANP), calcium-activated factor (CAF), kinase-activating

factor (KAF), receptor-transforming factor (Enzyme Committee of the International Union of Biochemistry, 1981). Two homogenous isozymes with different Ca^{2+}-sensitivities exist: calpain I and calpain II, active at μM or mM concentration of Ca^{2+}, respectively (Wheelock, 1982). It has been presumed to participate in various cellular functions mediated by Ca^{2+}, but their precise functions are not clear. They may be part of the general protein catabolic pathway in the cells. On the other hand, Mellgren (1987) has proposed that they may involve in the process of cell membrane fusion, the calcium-dependent proteolysis in postsynaptic membrane modeling in the hippocampus after long-term potentiation, and the activation of membrane-associated protein kinase C.

Busch et al. (1972) reported the isolation of a protein from skeletal muscle that causes Z-line removal from myofibrils in the present of Ca^{2+} at pH 7.0. Calpain has been purified from chicken, rabbit, porcine and human (Dayton et al., 1976a; Dayton et al., 1981; Imahori, 1982; Kawashima et al., 1984). The purified enzyme showed activities in removal of the Z-lines, partial degradation of the M-line, degradation of troponin, tropomyosin, and C-protein but no effect on myosin, actin, or α-actinin (Dayton et al., 1976b). This suggested that calpain may be responsible for myofibrillar protein degradation which must involve disassembly and proteolysis of the insoluble myofibrillar array at physiological pH. Both calpain I and calpain II were identified in (Wheelock, 1982) and purified

from (Dayton et al., 1981; Otsuka et al., 1988) skeletal muscle. Evidence shows that calpain I is the active form in skeletal muscle (Dayton et al., 1981; Otsuka et al., 1988).

In isolated skeletal muscles, protein breakdown can be stimulated by treatments that increase intracellular Ca^{2+} (Baracos et al., 1986). By using different classes of proteinase inhibitors, it was concluded that this effect is caused by the activation of a proteolytic pathway involving nonlysosomal thiol proteinase(s) (Ishiura et al., 1982; Zeman et al., 1985; Baracos et al., 1986; Furuno and Goldberg, 1986). Up to now, the only Ca^{2+}-dependent, cytosolic thiol protease known is calpain. It is quite possible that calpain is responsible for protein degradation in some pathological states in which the intracellular Ca^{2+} level is elevated through the damaged sarcolemma or the damaged sarcoplasmic reticulum because it was proposed that Ca^{2+} overload provides a general mechanism for cell necrosis in muscle disease (Joffe et al., 1981).

The theory of the involvement of calpain in myofibrillar protein degradation is supported by various reports. Calpain activity was found to be increased in muscle taken from human possessing Duchenne muscular dystrophy (Kar and Pearson, 1976), in the atrophying skeletal muscle of vitamin-E-deficient rabbits (Dayton et al., 1979), in denervated skeletal muscles (Elce et al., 1983) and in the tenotomized muscle of rat (Baker and Margolis, 1987). On the other hand, the electromicrographs

in several cases of diseased muscle of Duchenne muscular dystrophy, ischemia, and prune berry syndrome show intact M-line and suggesting that calpain but not the lysosomal proteinases is involved in the degradation process because only calpain degrade myofibrils at the Z-line without removing the M-line (Otsuka et al., 1988). However, essentially normal levels of calpain was found in patients with limb-gridle dystrophy and certain denervating diseases (Bird et al., 1980). It is not clear whether the elevation of intracellular Ca^{2+} level or the increase of calpain activity is responsible for the protein degradation in these disease states.

By using different proteinase inhibitors, it was illustrated that the basal degradative process in muscle did not involve lysosomal or thiol proteinases (Furuno and Goldberg, 1986).

By the finding that breakdown of average protein decreases in the presence of 2-deoxyglucose, an inhibitor of glycolysis or of azide, an inhibitor of oxidative phosphorylation, it is proposed that the breakdown of most proteins in myotubes seems to involve a proteolytic system requiring metabolic energy (Libby and Goldberg, 1981).

In reticulocytes, a ubiquitin-dependent, ATP-dependent proteolytic system has been described (Hershko and Ciechanover, 1982). An alkaline ATP-dependent protease that does not require ubiquitin has been described in soluble liver extracts (Beynon and Bond, 1986). But it is unlikely

that these systems are involved in muscles.

Moreover, it is now known that a ATP-dependent degradative system is present in all mammalian cells (Goldberg et al., 1982). A large ATP-dependent proteinase has been described in muscle cells, but the properties of this enzyme is not yet clear (Beynon and Bond, 1986).

A high-molecular-mass proteinase possessed three different hydrolytic activities termed "multicatalytic proteinase" was isolated from rat skeletal muscle (Dahlmann et al., 1985a). Fatty acids in the physiological concentrations activate this enzyme (Dahlmann et al., 1985b). It is suggested that, in vivo, potential activators such as fatty acids can induce the multicatalytic proteinase to participate in muscle protein breakdown. But it was reported that the amount and activity of muscle multicatalytic proteinase are not affected by starvation-induced hyperlipacidaemia (Dahlmann et al., 1987). Thus, the role of this enzyme is not yet clear.

Hydrolase H, a thiol enzyme exhibiting aminoendopeptidase activity and optimum pH at 7.5 to 8.0 (Okitani et al., 1981), and a heat-stable alkaline thiol proteinase exhibiting optimum pH at 8.0 at 60°C (Makinodan et al., 1988) were also described in skeletal muscle. But there is still not enough information to evaluate their role in muscle protein turnover.

1.5.2.3 Other Proteinases

In the past 30 years there have been a number of chymotrysin-like proteinases reported to be active in muscle homogenates at alkaline pH. Due to their optimum activity at alkaline pH, the proteinases are generally been referred to as "alkaline proteinases". Most of these enzymes are found to be serine proteinases (Bird et al., 1980).

It was found that alkaline proteinases were not localized in true muscle cells but in mast cells and did not contribute significantly to breakdown of average proteins in muscle (Libby and Goldberg, 1980). On the other hand, it was reported that purified serine proteinase actively degrades native myosin, actin, troponin, tropomyosin, α-actinin and M-protein from rabbit muscle (Yasogawa, 1978). Also, there are dramatic increases in the mast cell serine proteinases in muscular dystrophies (Katunuma et al., 1978; Sanada et al., 1978), starvation, and hormone imbalances (Bird et al., 1980). Thus, mast cell serine proteinases may involve in the accelerated or pathological degradation of myofibrillar proteins.

On the whole, we know only a little about the proteinases involved in the basal protein turnover. For the enhanced protein turnover in pathological states, we have identified some responsible candidates. However, their mechanism is still not clear.

1.6 Free Radicals and Their Scavenging Enzymes

1.6.1 Free Radicals and Their Damages

1.6.1.1 Free Radicals in Biological System

There are many ways to injure and kill cells. Amongst these is an important class of reactions dependent on the production of free radical intermediates to trigger an expanding network of multifarious disturbances.

Free radicals can be defined as molecules or molecular fragments with an unpaired electron (Slater, 1984). They are usually highly reactive and thus may cause tissue damage (Machlin and Bendich, 1987).

Intracellular free radicals are generated from the autoxidation and consequent inactivation of small molecules such as reduced flavins and thiols, and from the activity of certain oxidases, cyclooxygenases, lipooxygenases, dehydrogenases, and peroxidases. Oxidases and electron transport systems are the prime and continuous sources of intracellular reactive oxygenated free radicals (Fridovich, 1976; Machlin and Bendich, 1987). Electron transfer from transition metals such as iron to oxygen-containing molecules can also initiate free radical reactions. The sites of free radical generation encompass all cellular constituents including mitochondria, lysosomes, peroxisomes, and nuclear, endoplasmic reticula, and plasma membranes as well as sites within the cytosol (Machlin and Bendich, 1987).

Molecular species produced include, but by no means are limited to, hydroxyl, peroxy, hypochlorite, superoxide, and

alkoxy radicals, and reactive molecules such as hydrogen peroxide and singlet oxygen, which are not free radicals but are certainly reactive and capable of causing damage (Machlin and Bendich, 1987).

In almost all aerobic cells, the oxygen free-radicals are formed and they are mainly the source of intracellular free radicals (Halliwell and Gutteridge, 1984). They are the superoxide anion radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\cdot$).

$O_2\cdot^-$ is made during biological oxygen reduction, but the major biological sources remain unidentified. However, a large number of flavoprotein hydroxylases, oxidases and dehydrogenases can produce substantial amounts of $O_2\cdot^-$ (Fridovich, 1976). In the mitochondrial respiratory chain, $O_2\cdot^-$ is generated at two sites: the NADH dehydrogenase and the ubiquinone-cytochrome b area. Therefore $O_2\cdot^-$ may be produced as a by-product of oxidative metabolism, particularly in the abnormal state. It was proposed that the ubiquitous mitochondrial membranes is the most important physiological source of $O_2\cdot^-$ in animal cells (Turrens and Boveris, 1980). $O_2\cdot^-$ may cause the depolymerization of acid polysaccharides (Fridovich, 1976). However, $O_2\cdot^-$ itself does not appear to be directly cytotoxic but is able to initiate radical chain reactions (Halliwell and Gutteridge, 1984). The chain reactions are as the followings.

$$O_2\cdot^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

$$O_2\cdot^- + H_2O_2 \longrightarrow OH\cdot + OH^- + O_2 \qquad (2)$$

$$Fe^{3+} + O_2\cdot^- \longrightarrow Fe^{2+} + O_2 \quad (3)$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + 2H^+ + O_2\cdot^- \quad (4)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^- \quad (5)$$

$$O_2\cdot^- + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe salt}} OH\cdot + OH^- + O_2 \quad (6)$$

$$3H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe salt}} 2OH\cdot + O_2 + 2H_2O \quad (7)$$

Reaction (2) is referred to be Haber-Weiss reaction. But the rate constant for the reaction in aqueous solution is virtually zero. This reaction can only happen by the catalysis of transition metal ions. Reaction (5) is called the Fenton reaction. The combination of reaction (3) and (5) is an iron-catalyzed Haber-Weiss reaction and the net result is shown as reaction (6).

By the chain reaction induced by $O_2\cdot^-$, H_2O_2 and $OH\cdot$ are produced. Both of them are quite toxic to the cell. On the other hand, H_2O_2 alone can also produce $OH\cdot$ through the combination of reaction (4) ,(5) and (2) in the presence of iron salt. The net result are shown as reaction (7). In reaction (6) and (7), other transition metal ions, such as copper ion, may replace iron ion as the catalyst.

H_2O_2 is the most stable intermediate of the reduction of oxygen. It can be generated directly by divalent reduction of O_2 or indirectly by the univalent reduction of O_2 followed by dismutation of $O_2\cdot^-$. H_2O_2 is the primary product of the reduction of O_2 by numerous oxidases, such as urate oxidase (Halliwell and Gutteridge, 1984). Its

production has been demonstrated in suspensions of microsomes (membranes of the endoplasmic reticulum), illuminated chloroplasts, mitochondrial membrane, tissue homogenates, liver slices, and in whole perfused rat liver (Fridovich, 1976).It can also be produced by the peroxisomes (microbodies), cytosolic enzymes and the nucleus (Chance et al., 1979). The major sources of intracellular H_2O_2 may be endoplasmic reticulum and peroxisomes (Fridovich and Freeman, 1986).

It is believed that H_2O_2 is toxic, but it is not clear why it is so. H_2O_2 can cause the oxidation of sulfhydryl compound and of the methionyl residues in proteins. It can also cause the peroxidation of polyunsaturated fatty acids. However, these oxidations proceed at reasonable rates only at concentrations of H_2O_2 which exceed those attainable in vitro by at least four orders of magnitude. It seems possible that H_2O_2 is damaging, not by virtue of direct attack upon cellular components, but rather because it can, by reaction with $O_2\cdot^-$,i.e., through reaction (6), or with Fe^{2+}, i.e., through reaction (7), giving rise to the extremely reactive hydroxyl radical (Fridovich, 1976).

Hydroxyl radical ($\cdot OH$) is the most potent oxidant known (Fridovich, 1976). It reacts with extremely high rate constants with almost every type of molecule found in living cells: sugars, amino acids, phospholipids, DNA bases and organic acids (Halliwell and Gutteridge, 1984). In the biological system, it is mainly $O_2\cdot^-$ and H_2O_2 that are produced directly by the natural pathway. $O_2\cdot^-$ and H_2O_2 are

less reactive and can diffuse away from their sites of formation, leading to OH· generation in different parts of the cell whenever they meet a "spare" transition metal ion (Halliwell and Gutteridge, 1984).

1.6.1.2 Cell Injury Caused by Free Radicals

Reactive free radicals are able to produce chemical modifications of, and damage to proteins, lipids, carbohydrates and nucleotides (Slater, 1984). Therefore, if the reactive free radicals are produced *in vivo*, in amounts sufficient to overcome the normally efficient protective mechanisms, metabolic and cellular disturbances are expected to occur in various major ways.

If the reactive free radical is formed close to DNA, then it may produce a change in the structure resulting in a mutation or cytotoxicity. Oxidizing free radicals can alter the redox state of the NAD^+/NADH and $NADP^+$/NADPH couples and may produce NAD(P)· that can dimerize. Free radicals can also add to nucleotides to produce significant changes in their biological properties. Protein and non-protein thiol groups are readily oxidized by many free radicals and thiol radicals (S·) produced may dimerize and this type of free-radical-mediated disturbances of thiol groups may lead to profound changes in enzyme activity. Free radicals can also interact with cell components such as protein, lipid and nucleic acid, to form a stable covalently bound adduct that may grossly distort structure and function (Slater, 1984).

Reactive free radicals may also damage cells by membrane damage. They can covalently bind to membrane enzymes and/or receptors, thereby modifying the activities of membrane components. They can also covalently bind to membrane components and change their structure and produce effects on membrane function and/or antigenic character. Free radicals can also initiate of lipid peroxidation of polyunsaturated fatty acids (PUFA). In lipid peroxidation a primary reactive free radical interacts with a PUFA to initiate a complex series of reactions that result in a variety of degradation products: diene-bonded PUFA· free radical, lipid hydroperoxide, ethane, pentane, malonaldehyde (MAD), and some fluorescent and chemiluminescence materials. As a result, lipid peroxidation produce direct effects on membrane structure, and associated influences of the products of peroxidation on membrane fluidity, cross-linking, structure and function (Slater, 1984). Extensive lipid peroxidation can result in membrane disorganization.

1.6.2 Protection Against Free Radicals

1.6.2.1 The Protection Mechanism

Since oxygen free radicals are the species which are usually involved in the cells, protections are mainly concentrated on these species. Defenses against free radical damage include α-tocopherol (vitamin E), ascorbic acid (vitamin C), ß-carotene, glutathione (GSH), uric acid, bilirubin, and several metalloenzymes including glutathione peroxidase (GSH-peroxidase) (selenium), catalase (iron), and

superoxide dismutase (SOD) (copper, zinc, manganese) and protein such as ceruloplasmin (copper) (Machlin and Bendich, 1987).

Only three essential nutrients can directly scavenge free radicals. Vitamin E (α-tocopherol) is the major lipid-soluble antioxidant present in all cellular membranes and protects against lipid peroxidation. It can act directly with a variety of oxy radicals, including the peroxy radical (ROO·), CCl_3·, as well as with the superoxide radical (O_2·$^-$) (Machlin and Bendich, 1987). Vitamin E interrupts the chain reaction by reacting rapidly with the chain-propagating fatty acid radicals to yield α-tocopherol radical, which is unable to further propagate the chain reaction. It is particularly effective in this regard because it is an excellent antioxidant and because its hydrophobic properties cause it to partition into the biological membranes (Fridovich and Freeman, 1986).

Vitamin C is water soluble and, along with vitamin E can quench free radicals as well as singlet oxygen. Ascorbic acid has been shown to react directly with superoxide, hydroxyl radicals, and singlet oxygen. Ascorbic acid can also regenerate the reduced, antioxidant form of vitamin E (Machlin and Bendich, 1987).

β-carotene, a pigment found in all plants, is the most efficient quencher of singlet oxygen known in nature and can also function as an antioxidant. It has been found in cellular membranes,including those of lysosomes (Machlin and

Bendich, 1987).

There are, in addition, several nutritionally essential minerals incorporated into protective antioxidant enzymes. Zinc, copper, and manganese are required for the activity of the two types of SODs. Selenium is an essential component of GSH-peroxidase. Iron involves in the activity of catalase, a hemoprotein. GSH is a tripeptide composed of nonessential amino acids and involved in the function of GSH-peroxidase (Machlin and Bendich, 1987).

The antioxidant enzymes function mainly intracellularly. Therefore, extracellular free radicals must be inactivated by the circulating antioxidants such as vitamins discussed as well as by ceruloplasmin, a copper-containing protein (Machlin and Bendich, 1987).

In skeletal muscles, Boldyrev et al. (1988b) suggested that histidine-containing dipeptides, carnosine (ß-alanyl-L-histidine) and its methyl derivatives, anserine, play the antioxidative role. They believed that skeletal muscle dipeptides are involved in the regeneration of the active form of α-tocopherol and serve as an effective "antioxidant buffer" which protects muscle cell membranes from uncontrolled lipid peroxidation. They proposed that skeletal muscle used these dipeptides rather than glutathione because that NADPH was needed for the reduction of glutathione disulfide (GSSG) to GSH and muscle lacked of NADPH-regenerating enzymes (Boldyrev et al., 1988a).

1.6.2.2 Free Radical Scavenging Enzymes

The primary defense in the cell against free radical damage is provided by enzymes that catalytically scavenge the intermediates of oxygen reduction. There are cytochrome oxidase, SOD with either iron (FeSOD) or manganese (MnSOD) at the active site, and others with both copper and zinc (CuZnSOD). FeSODs and MnSODs are characteristic of prokaryotes, while CuZnSODs are characteristic of eukaryotes. Eukaryotes generally contain both CuZnSOD and MnSOD. There are also catalases that are hemoproteins, and others, found in organisms incapable of heme synthesis, that may be flavoproteins. There are heme-containing peroxidases that can utilize a wide variety of electron donors for the reduction of H_2O_2, and others that contain selenium and specially utilize reduced GSH as the reducing substrate (Fridovich, 1978).

The first line of defense is simply avoidance of the univalent pathway by enzymes with multiple electron-carrying components, which can accomplish the tetravalent reduction of dioxygen to water without the release of intermediates. Cytochrome oxidase is such an enzyme, and in actively respiring cells such as lung cell, it accounts for more than 90% of the observed dioxygen reduction without releasing $O_2\cdot^-$ or H_2O_2 as intermediates. This strategy of avoidance of the univalent pathway reduces the burden of reactive intermediates the cell must face (Fridovich and Freeman, 1986).

The second line of defense is provided by SOD. $O_2\cdot^-$ is

cleared by SOD through the dismutation reaction. SODs catalyze the reaction

$$2O_2\cdot^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

and do so with an efficiency that approaches the theoretical diffusion limit (Fridovich and Freeman, 1986).

Catalase removes H_2O_2 by converting it to water and oxygen

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

and functions as the third layer of antioxidant defense. Indeed, catalase can act as a peroxidase when the concentration of H_2O_2 is kept low and electron donors such as alcohols or formate are present (Oshino et al., 1973; Fridovich, 1976). GSH-peroxidase, as well as other peroxidases, decomposes H_2O_2 and lipid peroxides. It is thought to play a major role as scavengers of H_2O_2 under low endogenous production rates of H_2O_2 pertaining under physiological conditions contributed by the generation within endoplasmic reticulum (Jones et al., 1981). The relative contributions of catalase and GSH-peroxidase in decomposition of endogenously generated H_2O_2 are determined largely by the subcellular localizations of the enzymes in the cells (discussed later) and the lower Km of GSH-peroxidase for H_2O_2 (Jones et al., 1981). However, catalase becomes more important because the catalytic reaction, which

has a greater rate constant, predominates over the peroxidation reaction (Fridovich and Freeman, 1986). Glutathione-reductase GSSG-reductase), which catalyzes the reduction of the GSSG by NADPH nd prevents depletion of cellular glutathione thiol, is also thought to be involved in these processes (Fridovich and Freeman, 1986).

It is clear that efficient removal of the first two intermediates of oxygen reduction, $O_2\cdot^-$ and H_2O_2 will prevent formation of the third, OH·. All these enzymes remove $O_2\cdot^-$ and H_2O_2 in the cell. This is fairly rational, since OH· reacts avidly with many substances and its specific enzymatic scavenging would be impossible (Fridovich, 1978).

These enzymes have different locations in the cell. CuZnSOD is found predominantly in cytosol, while MnSOD is found mainly in mitochondria (Asayama et al., 1986). GSH-peroxidase is a cytosolic enzyme (Jones et al., 1981). In many cases, catalase is localized in subcellular organelles such as the peroxisomes (microbodies) of liver and kidney or in much smaller aggregates, such as the microperoxisomes found in a variety of other cells (Stauber and Bird, 1974; Chance et al., 1979).

1.6.3 Involvement of Free Radicals in Various Diseases

Free radical pathology is the result of uncontrolled or abnormal radical reactions occurring in the cells. The extent of tissue damage is the result of the balance between the free radicals generated and the antioxidant protective

defense system.

Some diseases are suggested to be related to free radicals. Continuous exposure to free radical-containing environmental pollutants has been associated with lung damage, emphysema, and cancer (Fridovich and Freeman, 1986). Colon cancer, precancerous dysplasias of the cervix and intestine are also found to be related to the free radicals. Cardiovascular diseases including atherosclerosis and the tissue injury after myocardial infraction have been shown to result in part from free radicals generated at the site of damage. In addition, the formation of cataracts, the photodermatoses, inflammatory diseases including arthritis, and the aging process itself have all been associated with free radical damage (Machlin and Bendich, 1987).

1.6.4 Involvement of Free Radicals and Their Scavenging Enzymes in Skeletal Muscle and Myopathy

Considerable quantities of peroxides were measured in many tissues, including skeletal muscles, with the depletion of vitamin E (Bieri and Anderson, 1960). This showed that free radicals can be formed in muscle. Muscle tissue free radicals originate primarily in mitochondria. They are related to flavoprotein and ubiquinone-semiquinone radicals (Nagaoka et al., 1981). The free radical concentration in muscle was shown to be associated with the rate of electron flow in the respiratory chain, which is regulated by the mitochondrial metabolic states (Koren et al., 1983). As a result, the concentration of free radical is greatly

increased by exercise and thus may cause limited damage to mitochondrial membranes which, in a chronic training situation, may be the initiating stimulus to mitochondrial biogenesis (Davies, 1982).

Lipid peroxidation is known to be increased in nutritionally induced animal myopathies (Bieri and Anderson, 1960). The lipid peroxides, measured as thiobarbituric acid-reactive products, were reported to increase in muscles of chicken, mice (Omaye and Tappel, 1975) and human (Kar and Pearson, 1979) with muscular dystrophies. Lipid peroxidation of the red blood cells was found to be significantly higher in Duchenne muscular dystrophy patients (Matkovics et al., 1982).

For the free radical scavenging enzymes, their activities has been investigated in many forms of myopathies. Kar and Pearson (1979) reported elevated levels of catalase and GSSG-reductase activities in human muscular dystrophies. In animal dystrophies, Omaye and Tappel (1975) reported increased activities of GSH-peroxidase and GSSG-reductase in dystrophic chickens and mice. Park et al. (1979) reported increases in activities of GSH-peroxidase, GSSG-reductase and SOD in avian muscular dystrophy. All of these findings suggest increased turnover of oxygen species in these muscular dystrophies. Mizuno (1984) found increased activities in SOD in early stage of the development and suggested the presence of increased turnover of active oxygen species from the early stage of the disease

in avian muscular dystrophy and the involvement of oxygen species in pathogenesis of this disorder.

In denervated rat muscles, Asayama et al. (1986) found that the CuZnSOD activity decrease slightly in the slow but not in fast muscle. Also, activities of MnSOD, cytochrome c oxidase, GSH-peroxidase decrease only in the slow muscle. From these result, free radicals do not seems to involve in pathogenesis of denervation myopathy.

By a systematic study of free radical scavenging enzymes in neuromuscular disease, Burr et al. (1987) showed that muscles involving in motor neuron disease demonstrated markedly decrease MnSOD concentrations and increase GSH-peroxidase activity with normal CuZnSOD activity. The result of SOD is similar to that of Asayama et al. (1986) but that of GSH-peroxidase is just opposite. In muscle of primary polymyositis patients, CuZnSOD, GSH-peroxidase and catalase activities decrease markedly. For myotonic dystrophy patients, their muscles exhibit no change from the control muscle in these enzymes. These studies indicate disease-specific differences from the normal in redox-active enzymes in DMD and other neuromuscular disorders. This raises questions regarding the control of redox metabolism in muscle, but does not permit formation of a definitive hypothesis regarding pathogenesis (Burr et al., 1987).

The contribution of free radicals and the related enzymes in myopathy is not yet clear. By the above result, it seems that in those involved in the failure of motor neuron, the oxygen species do not actively participate in

the pathogenesis. However, Stauber et al. (1977) demonstrated elevated catalase activities in denervation or muscle wasting caused by other factors and suggested that the specific activities of catalase in muscle homogenates may be a valid indicator of the presence of skeletal muscle wasting. This shows the possible ubiquitous involvement of catalase in myopathy. Also, high specific activities of catalase are found in tissues that readily oxidize lipids, such as red muscles. Therefore, the role of catalase in skeletal muscle is waiting to be clarified.

1.7 Altered Physiological States of Skeletal Muscle

One of the reasons for studying muscle is to look into the sometimes-fatal neuromuscular diseases that occur in human.

Apart from the well-known temporary disorders in muscle such as cramp, "stitches", and general, sometimes painful muscular fatigue associated with lactic acid accumulation, various distinct categories of long-term muscular or neuromuscular disorders have been identified.

Two broad divisions occur in the description of myopathies. One division includes the dystrophies that are inherited, and the other includes the defects acquired after birth.

Inherited myopathies include the classic muscular dystrophies such as Duchenne dystrophy, Becker muscular dystrophy, congenital muscular dystrophy, limb-gridle

dystrophy, myotonic dystrophy and biochemical disorders such as glycogen storage diseasse and lipid storage diseases. Also in this category are the diseases clearly associated with motor neuron defects such as the spinal muscular atrophies.

Acquired myopathies include three broad types: inflammatory, noninflammatory, and traumatic myopathies. Inflammatory myopathies can either be idiopathic in origin or due to infection from extrinsic agents (viruses, bacteria, parasites). The idiopathies include polymyositis and sarcoidosis. Extrinsic inflammatory myopathies include the problems associated with viruses such as the influenza virus.

Noninflammatory acquired myopathies can arise from a host of idiopathic problems, such as endocrine disease (thyrotoxic myopathy) and carcinomatous myopathy. But they can also arise from extrinsic sources, such as drugs or alcohol. The over-reactivity syndromes such as cramp, tetany, contracture, etc. are also included in this category. There are also two bacterial toxin caused non-inflammatory myopathies, botulism and tetanus.

A special example of acquired myopathy is the disorder due to physical damage or trauma. The well-known disease myasthenia gravis is classified as a disease of the neuromuscular junction (Squire, 1986).

In many forms of myopathies, it is not known whether the very obvious structural or biochemical abnormalities in

the muscle are the primary defect or whether these are symptoms or consequences of a defect elsewhere in the same motor unit. The studies of denervation model may provide a clue to this problem. Also, the studies of systems which may be involved in cell damage or cell component degradations would provide an insight into the pathogenic mechanism.

1.8 Objectives of the Present Study

In this study, enzyme activities in two models of myopathy were investigated. They are muscle atrophy caused by alcohol administration and that resulted from denervation. The enzymes examined belongs to mainly three systems. They are energy metabolizing enzymes, lysosomal enzymes and free radical scavenging enzymes. The objective of this study is to characterize the biochemical events during muscle atrophy following ethanol administration and surgical denervation. During the denervation atrophy studies, the effect of clenbuterol was also investigated to see whether the denervation induced muscle atrophy could be reversed by ß-agonist administration. If muscle atrophy can be reversed, can the effect be linked to the biochemical parameters measured?

CHAPTER 2 METHODOLOGY

TABLE 2-1

Chemicals Used and Their Suppliers

Chemicals	Suppliers	Cat. No.
2-Mercaptoethanol	Sigma	M-6250
Acetic Acid	Reidel-deHaën	33209
Albumin, Bovine Serum	Sigma	A-9647
ATP	Sigma	A-3377
Azide, Sodium Salt	Merck	6688
Brij 35	Sigma	430AG-A
Butanol	BDH	27500
Catalase	Sigma	C-10
Citric Acid	Sigma	C-7129
Coomassie Brilliant Blue G	Sigma	B-1131
Cyanide, Potassium Salt	Merck	4967
Cysteine	Sigma	C-7755
EDTA	Sigma	ED2SS
Epinephrine	Sigma	E-1635
Ethanol	Reidel-deHaën	32221
Fast Garnet GBC Base	Sigma	F-8504
Folin-Ciocalteus Phenol Reagent	Merck	9001
Glucose-6-phosphate Dehydrogenase	Sigma	G-5760
D-glucose Monohydrate	Reidel-deHaën	16301
Haemoglobin	Sigma	H-2625
Hydrogen Peroxide	Merck	7209

...Continued

TABLE 2-1 (Continued)

Chemicals Used and Their Suppliers

Chemicals	Suppliers	Cat. No.
Magnesium Acetate	Merck	5819
Mersalyl Acid	Sigma	M-8000
NADH	Sigma	N-6005
$NADP^+$	Sigma	N-3886
2-naphthylamine	Sigma	N-8381
P-nitrophenol	Sigma	104-8
P-nitrophenyl Phosphate	Sigma	N-3254
Oxaloacetic Acid	Sigma	O-4126
Perchloric Acid	Merck	519
Pyruvic Acid	Sigma	P-2256
Superoxide dismutase	Sigma	S-2515
Tartrate, K-Na salt	Ajax	503
Thiobarbituric acid	Sigma	T-5500
Tris	Sigma	T-1378
Z-Arg-NNap	Sigma	B-4750

2.1 Materials

The chemicals used and their suppliers are listed in Table 2-1. All chemicals are of analytic grade.

2.2 Animals

Sprague-Dawley rats originated from Charles River Laboratory (Japan), born and raised in our animal care unit, were used. All animals were housed in wire-bottomed cages, acclimated at a room temperature of 22°C with alternate cycles of 12 hours of light and darkness, and were provided with Purina rat chow and tap water.

Animal treatment protocol rats will be described in detail in the corresponding chapters.

2.3 Muscles Used in This Study

The muscles of the rat hind limbs were the targets of this study. These included gastrocnemius, extensor digitorum longus (EDL), soleus, and tibialis anterior (TA). Figure 2.1 is an anatomical picture which shows the arrangement of these muscles in the lower leg of rats.

Gastrocnemius and TA are muscles having type I and type II fibers (details about these fibers have been described in chapter 1) in similar amount (Close, 1972) and are treated as mixed type muscles. EDL contains mainly type II fibers (90% type IIa and 10% type I) (Squire, 1986) and is taken as representative of fast-twitch muscle. Soleus

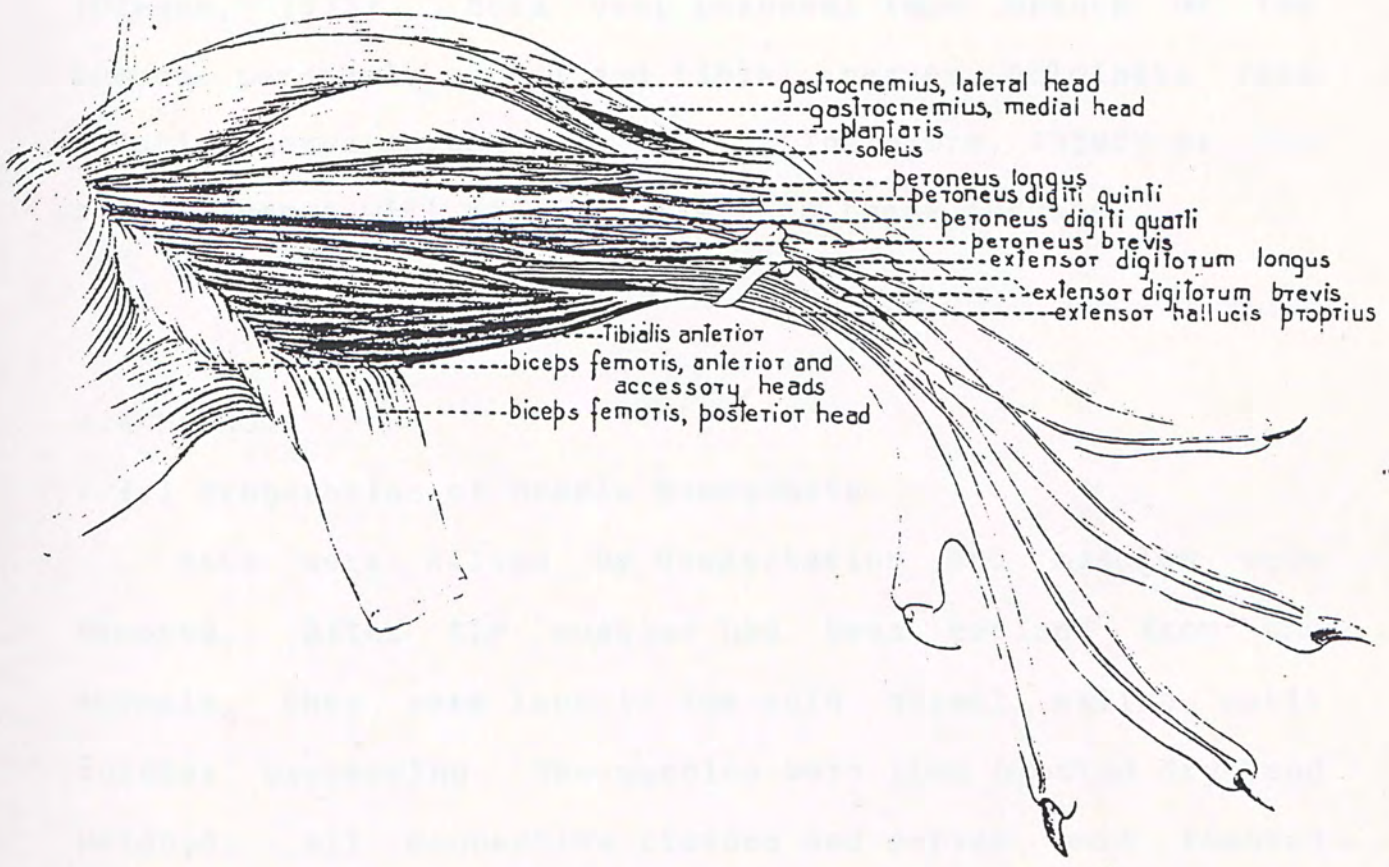

Figure 2.1 Anatomy of the hind-leg muscle of rat. (From Greene, E.C.: "Anatomy of the rat" American Philosophical Society, 1935)

consists of mainly type I fibers (85% type I and 15% type IIa) (Squire, 1986) and is treated as a slow-twitch muscle (Asayama et al., 1986).

EDL and TA are innervated by deep peroneal nerve, while gastrocnemius and soleus are innervated by tibial nerve (Greene, 1935). Both deep peroneal (one branch of the common peroneal nerve) and tibial nerves originate from sciatic nerve (Bruck-Kan, 1979). Therefore, injury of the sciatic nerve will have an effect on these muscles.

2.4 Methods

2.4.1 Preparation of Muscle Homogenate

Rats were killed by decapitation and muscles were removed. After the muscles had been excised from the animals, they were kept in ice-cold normal saline until further processing. The muscles were then blotted dry and weighed. All connective tissues and nerves were removed with scissors, and the muscles were minced and homogenized with a Polytron (3 X 30 sec with 30 sec intervals) in 9 volumes of ice-cold 50 mM Tris (pH 7.4 at 20°C). As a result, 10% crude homogenates were prepared. The crude homogenates were then further homogenized in a Teflon-pestle homogenizer by five strokes, followed by sonication (3 X 15 sec with 10 sec intervals). The debris of the crude homogenate was removed by low speed centrifugation (800 g, 10 minutes). The clear supernatants were used for all the assays except for the determination of MAD. All procedure

were done at 4°C. The muscle homogenates were then frozen quickly in liquid nitrogen and kept at -70°C until thawing for the various assays.

2.4.2 Protein Assays

Protein content of muscle homogenate was determined using the method modified from that described by Lowry et al. (1951). The samples were first digested in 1 M NaOH at room temperature for 20 minutes, then 0.1 ml of digested protein was added to 1.0 ml of reagent C. After standing at room temperature for 10 minutes, 0.1 ml of 1 M Folin-Ciocalteus phenol reagent was added. The absorbance at 750 nm was measured after standing the final mixture at room temperature for a further 45 minutes. Reagent C was prepared by mixing 100 ml 2% Na_2CO_3 in 0.1 N NaOH with 1 ml 1% $CuSO_4 \cdot 5H_2O$ and 1 ml 2% K-Na Tartrate. BSA was used as the protein standard.

2.4.3 Lipid peroxide Assays

Malonaldehyde (MAD), one of the major secondary oxidation products of peroxidized polyunsaturated fatty acids, was chosen as the representative of lipid peroxide. Its level in both liver and skeletal muscle were determined, using 2-thiobarbituric acid (TBA) as the reagent.

The TBA test for rat liver samples was performed by the method of Csallany et al. (1984). One gram of rat liver was

homogenized in a Polytron homogenizer to make a 10% homogenate with distilled water. A 0.5 ml aliquot of the homogenate was added to 3 ml 1% phosphoric acid and 1 ml 0.8% TBA solution. The mixture was heated for 45 minutes in boiling water. After cooling, 4 ml of n-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation at 800 g for 10 minutes. The absorbance of the butanol phase was measured at both 532 nm and 520 nm. The difference between the two readings was used to calculated the MAD level.

The MAD measurement method in muscle was modified from that of Csallany et al. (1984). One g sample was homogenized in a total volume of 5 ml distilled water in a polytron homogenizer for 30 s. Then 5 ml of 10% $HClO_4$ was added and the homogenate was centrifuged to precipitate the acid insoluble protein. The clear supernatant (4 ml) was added to 1 ml of 0.8% TBA reagent and incubated for 90 minutes in an 80°C water bath. Absorbance at 532 nm was measured .

It should be noted that the muscle homogenate used in this assay was different from that described in section of 2.4.1.

2.4.4 Enzyme Assays

2.4.4.1 Acid Proteinases

The method for the determination of acid proteinase was modified from that described by Maskrey et al. (1977). The

enzyme activity was measured by incubating the sample for 2 hours in a final volume of 0.5 ml containing a 50 mM CH_3COONa/CH_3COOH buffer, (pH 3.5) and 3 mg haemoglobin. A quantity of homogenate equivalent to 5 mg wet weight of muscle was used. $HClO_4$ (1 ml 10%) was then added and, after removing the precipitated protein by centrifugation (800 g, 20 min), the absorbance of the clear supernatant was measured at 280 nm. The value obtained was corrected by subtracting the blank in which the homogenate was added at the end of the incubation. An absorbance of 1.000/2h is taken as 1 unit of activity. The hemoglobin had been denatured before use by keeping it at pH 1.3 and 37°C for 1h and subsequently dialyzed overnight in the cold against the 50mM CH_3COONa/CH_3COOH buffer. (pH 3.5). The concentration of haemoglobin in the dialysate was measured by the method described of Bradford (1976) with haemoglobin as standard, and then diluted to the appropriate concentration.

2.4.4.2 Cathepsin B (EC 3.4.22.1)

The activity of cathepsin B was assayed by the method of Turk and Kregar (1984) and that of Barrett (1972) with some modifications. Using Z-Arg-NNap as the substrate, the principle of the assay is represented by the following equation :

$$\text{Z-Arg-NNap} + H_2O \xrightarrow{\text{Cathepsin B}} \text{Z-Arg} + \text{2-naphthylamine}$$

2-naphthylamine is released. The amount of 2-naphthylamine

released per unit time is determined colorimetrically by coupling with a diazonium salt, Fast Garnet, and is therefore a measure of cathepsin B activity.

In the assay, homogenate corresponding to 25 mg wet weight muscle was incubated in 1 ml of 73 mM of potassium phosphate buffer (pH 6.0), 1 mM EDTA, 2 mM cysteine, and 0.5 mM Z-Arg-NNap, at 40°C for 4 hours. Then the color reagent was added to stop the reaction and to develop the color. The color reagent was prepared by mixing 1.0 ml diazonium salt solution with 0.1 ml 0.2 M $NaNO_2$ in ice for 5 minutes and then diluted with 100 fold of Mersalyl-Brij solution. Diazonium salt solution is a 10 mM solution of 4'-amino-2,3'-dimethylazobenzene in ethanol and water. Mersalyl-Brij solution is a water solution containing 5 mM of mersalyl acid, 0.8 mM EDTA and 2% of Brij 35 with a pH value of 4.0.

The color product produced was then extracted with butanol and the amount of 2-naphthylamine was determined by measuring the absorbance at wavelength 520 nm and calibrating from the standard curve. A standard curve of 2-naphthylamine was prepared by replacing muscle homogenate in the assay with 2-naphthylamine in different concentration.

One unit of cathepsin B is defined as the activity which release 1 nmole of 2-naphthylamine per minute.

2.4.4.3 Acid Phosphatase (EC 3.1.3.2)

The modified Moss (1984) method was adopted. The assay

is based on the following reaction :

$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow[\text{pH 4.9}]{\text{acid phosphatase}} \text{p-nitrophenol} + Pi$$

$$\text{p-nitrophenol} \xrightarrow[\text{pH 11}]{\text{dissociation and rearrangement}} \text{p-nitrophenolate ion}$$

Hydrolysis of p-nitrophenyl phosphate by acid phosphatase at pH 4.9 liberates p-nitrophenol. The reaction is stopped by raising the pH to 11 by addition of NaOH. At this pH the strongly-colored quinonoid p-nitrophenolate ion is produced and its absorbance is measured at 405 nm.

The assay mixture contained 6.3 mM p-nitrophenyl phosphate, 37.5 mM citrate, 8 mM Tris at pH 4.9, and homogenate equivalent to 5 mg wet weight muscle. After incubating at 37°C for 10 minutes, NaOH which would give a final concentration of 77 mM was added. Absorbance at 405 nm was then measured. A standard curve of p-nitrophenol was obtained by substituting the muscle homogenate with a p-nitrophenol solution. One Unit of acid phosphatase activity is defined as the release 1 nmole of p-nitrophenol per minute at 37°C.

2.4.4.4 Catalase (EC 1.11.1.6)

Catalase activity was measured by the breakdown of H_2O_2. :

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The method used was based on that of Aebi (1983). Homogenate corresponding to 2 mg wet weight muscle was added to a 10.5 mM solution of H_2O_2 in 50 mM phosphate buffer (pH 7.0) at 25°C. The final volume of the assay was 1.0 ml. The degradation of H_2O_2 was monitored by measuring the decrease in absorbance at 240 nm since H_2O_2 gave high absorption at this wavelength. One unit of catalase activity was defined as those decompose 1 µmole of H_2O_2 per minute.

To verify that the activity measured was that of catalase, a control containing azide (NaN_3), an inhibitor of catalase, in a final concentration of 1 mM was used (Theorell and Ehrenberg, 1952; Cederbaum, 1987). The difference with and without azide was interpreted as the actual activity.

2.4.4.5 Superoxide Dismutases (EC 1.15.1.1)

Superoxide dismutase (SOD) is a family of metalloenzymes which is known to accelerate the spontaneous dismutation of the superoxide radical ($O_2\cdot^-$) to hydrogen peroxide (H_2O_2) and molecular oxygen. The assay is based on the ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 (Misra and Fridovich, 1972). The production of adrenochrome in a final volume of 1 ml containing 0.3 mM epinephrine, 0.1 mM EDTA, and 50mM sodium carbonate buffer (pH 10.2) at 30°C was followed at 480 nm. One unit of the enzyme is the amount of SOD capable of

inhibiting 50% the rate of epinephrine oxidation observed in the control in which no enzyme was added.

To discriminate the activity of CuZnSOD from that of MnSOD, KCN in a final concentration of 2mM was included in the assay mixture, as described by Weisiger and Fridvoch (1973) and Mizuno (1984). Those insensitive to cyanide was MnSOD while the other was CuZnSOD. In the absence of cyanide, the activity measured was total SOD.

2.4.4.6 Malate Dehydrogenase (EC 1.1.1.37)

The method adopted to measure the activity of malate dehydrogenase (MDH) was based on Boehringer Mannheim GmbH (1973). The assay utilize the following reaction which is catalyzed by MDH.

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+$$

Homogenate equivalent to 0.2 mg wet weight muscle was added to a mixture containing 0.1 M phosphate buffer (pH 7.5), 0.5 mM NADH, and 1 mM oxaloacetate. The change in absorbance at 340 nm at 25°C was measured. One unit MDH activity is defined as the amount of enzyme required to oxidize 1 µmole of NADH per minute at 25°C.

2.4.4.7 Lactate Dehydrogenase (EC 1.1.1.27)

Assay of lactate dehydrogenase (LDH) utilizes its ability to catalyze the following reaction.

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

LDH activity was determined by the rate of oxidation of NADH. The procedure used was modified from that of Vassault (1983). The final mixture was in a volume of 1.1 ml and was consisting of 65 mM Tris-HCl buffer (pH 7.4), 0.3 mM NADH and homogenate equivalent to 1 mg or 0.5 mg wet weight of muscle. Enzyme measurement was made by continuously monitoring the rate of change in absorbance at 340 nm at 25°C. One unit of LDH activity is able to catalyze the oxidation of 1 µmole of NADH per minute at 25°C.

2.4.4.8 Hexokinase (EC 2.7.1.1)

The following reactions are utilized for the determination of hexokinase activity.

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{Glu-6-P} + \text{ADP}$$

$$\text{Glu-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P dehydrogenase}} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^+$$

The procedure described by Brooks (1976) was adopted. The final reaction mixture contained 50mM Tris-HCl buffer (pH 7.4), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM ATP, 0.4 mM $NADP^+$, 1 mM D-glucose, 4 unit of glucose-6-phosphate dehydrogenase (G6P). The final volume of the assay mixture was 1.2 ml. Change of absorbance at wavelength 340 nm at 25°C was recorded to calculate the

enzyme activity. One unit enzyme reduces 1 µmole of $NADP^+$ per minute at 25°C.

2.4.4.9 Alkaline Phosphatase (EC 3.1.3.1)

Alkaline phosphatase hydrolyzes p-nitrophenyl phosphate, giving p-nitrophenolate ions, which show absorption at 405 nm, at alkaline condition.

$$\text{p-nitrophenylphosphate} + H_2O \xrightarrow[\text{pH 10.5}]{\text{Enzyme}} \text{p-nitrophenolate ion} + Pi$$

The method of Boehringer Mannheim GmbH (1973) was adopted. Homogenate equivalent to 10 mg wet weight of muscle was added to a mixture of 98.4 mM glycine buffer (pH 10.5), 1 mM Mg^{2+}, 0.1 mM Zn^{2+}, and 6 mM nitrophenyl phosphatase. The change in absorbance at 405 nm was monitored. Increase of absorbance at 405 nm contributed by p-nitrophenolate ion per unit time is a measure of the enzyme activity. One unit is defined as the activity which degrades 1 nmole of p-nitrophenylphosphate and therefore the formation of 1 nmole of p-nitrophenolate ion per minute at 25°C.

CHAPTER 3 EFFECT OF ETHANOL ADMINISTRATION ON SKELETAL MUSCLES : A STUDY OF ACUTE ALCOHOLIC MYOPATHY

3.1 Introduction

Alcohol is the oldest and the most common psychoactive drug used throughout the world. Moderate drinking of alcohol has been reported to have a number of medical benefits (Singer, 1972). For example, alcohol can promote circulation and the production of blood, as well as promoting digestion and appetite. Traditional Chinese medical beliefs also assert that alcohol has curative effects on rheumatism and anemia, especially for women after childbirth. However, alcohol has the potential of being abused.

Alcohol abuse brings about a large number of problems which affect the person himself, his family and even the whole community. Among the problems, the most obvious one is the physical damages inflicted by alcohol to the body. Various organs and tissues in the body can be affected by ethanol. Liver, the main site of ethanol oxidation, displays the broadest spectrum of metabolic response and disease as a consequence to ethanol abuse (Lieber and DeCarli, 1977). Other tissues, including the brain (Noble and Tewari, 1977), gut (Baraona and Lindenbaum, 1977), heart (Bing and Tillmanns, 1977), endocrine systems (Gordon and Southren, 1977), bone (Saville, 1977), blood (Lindenbaum, 1977) and muscle (Geller and Rubin, 1977), can also be severely affected and damaged by ethanol.

In this chapter, the acute effect of ethanol on skeletal muscles is studied.

It takes a long time for human to recognize the

relation between alcohol abuse and myopathy. In 1822, James Jackson in commenting on a 'particular disease resulting from the use of ardent spirits' described a progressive weakness of the limbs. Magnus Huss, in 1849, described muscular weakness independent of a clinical neuropathy in chronic alcoholics. Gudden, in 1896, described three alcoholics in whom histological changes suggested myopathy. For the half century thereafter, this disease drew no attention (Geller and Rubin, 1977; Martin et al., 1982).

Recent interest stems from the report of Hed et al. (1955) of a 53-year-old alcoholic who developed sudden pain, tenderness and swelling of the legs. This condition resolved spontaneously, but recurred several times during the next 3 years. The patient died 4 days after the onset of severe calf pain and myoglobinuria. At autopsy the muscle fibers of the calf were swollen and fragmented, had lost their striations, and were infiltrated by polymorphonuclear leukocytes. Hed's group then went on to suggest a causal relationship between alcohol ingestion and acute myonecrosis in a series of 12 patients (Hed et al., 1962). Varying degrees of muscle damage were seen in muscle biopsies and severe rhabdomyolysis could be demonstrated in all of the muscles studied at autopsy. For this reason, this syndrome is sometimes referred to be alcoholic rhabdomyolysis.

Ekbom et al. (1964) described a rather different

syndrome of progressive muscle wasting and weakness in chronic alcoholics, but without the acute features described by Hed.

Several forms of alcoholic myopathy have been recognized in recent years. They are the subclinical, acute, and chronic alcoholic myopathy (Geller and Rubin, 1977). Acute alcoholic myopathy is an acute syndrome of muscle pain, tenderness, and edema occurring after acute excess alcoholic ingestion. Chronic myopathy is a gradual, insidious development of muscle wasting and weakness without pain or tenderness (Smith, 1977). Although the chronic myopathy is probably much more common than the acute one, the latter is accompanied by myoglobinuria and may even progress to death.

For a long time, acute alcoholic myopathy has not been studied extensively because it is not a common disease and therefore samples are limited. In addition, lacking of proper control is reason : factors other than alcohol ingestion may be involved.

In 1980, Haller and Drachman reported an experimental model of acute alcoholic myopathy in rats by a combination of prolonged exposure to ethanol and a brief period of food deprivation. Their idea came from the fact that exposure to high concentration of alcohol is commonly associated with poor nutrition. This provided an opportunity to study the pathology of this disease. Haller (1985) had examined the histological features of muscle injury and repair in this experimental model and found that the features in this model

closely parallel those in human acute alcoholic myopathy.

To study the biochemical changes in myopathy, we have adopted the model of Haller (1985) with some modifications.

Muscular atrophy represent an increase in degradation or a decrease in synthesis of cell components, usually the structural proteins. Warnes et al. (1981) had reported increased rates of myofibrillar protein breakdown in a variety of muscle-wasting diseases and these diseases may respond to therapy directed towards an inhibition of muscle protease activity. Therefore, the proteinase activities in alcoholic myopathy were studied to see if increase in proteolytic activities correspond to the atrophy.

It had been shown that ethanol may induced lipid peroxidation in the liver (Shaw et al., 1981). Lipid peroxidation may cause cell damage (Slater, 1984). Lipid peroxides were reported to increase in muscles of chicken, mice (Omaye and Tappel, 1975) and human (Kar and Pearson, 1979) with muscular dystrophies. Also, the activities of free radical scavenging enzymes were found to be changed in many form of muscle atrophies (Omaye and Tappel, 1975; Stauber et al., 1977; Kar and Pearson, 1979; Park et al., 1979; Mizuno, 1984; Asayama et al., 1986 and Burr et al., 1987). However, the involvement of free radicals in alcoholic myopathy has not been reported. To clarify the role of free radicals in alcoholic myopathy, the activities of the component enzymes of the free radical scavenging system were investigated.

Ethanol has been reported to cause metabolic changes in various tissues. Since muscle does not metabolize ethanol (Martin et al., 1982), the metabolic changes in the alcoholic myopathy may be a consequence of other changes or damages. By comparing the changes in this disease with those in the other disease states, much information may be obtained.

3.2 Materials and Methods

3.2.1 Materials

Materials used in the various assays have been described in chapter 2. The sources of the chemicals involved in the treatment of animals are as follows : Ethanol (A.R. grade) from James Burrough Ltd., saccharin from BDH, sucrose from the supermarket.

3.2.2 Animal Treatment Regimen

The treatment of rats was based on the protocol of Haller (1985) with some modifications. Male Sprague-Dawley rats of age 60±5 days with similar body weight were divided into two groups. One was the experimental (ethanol-fed) group while another was the pair-fed control group. Each rat was housed in individual cage. For the experimental group, ethanol solution was given as the sole drink. In order to make the rats adapt to the ethanol treatment, a special procedure was used. The initial concentration of

ethanol was 5% (v/v) in tap water and then increased by 5% in 3 day intervals until a concentration of 30% was attained and this concentration was maintained thereafter. The amount of ethanol intake by the rats increased from about 3g to 14g each day per Kg body weight (see Figure 3.1). Saccharin at a concentration of 0.05mg/ml was also added in the ethanol solution to attract the rat to take the ethanol solution. In this period, rat chow was provided ad libitum.

For the pair-fed control rats, an amount of sucrose solution calorically equivalent to the ethanol consumed by the experimental group in the previous day were provided as the extra drink besides tape water. The supply of rat chow was limited to the same amount consumed by the experimental group in the previous day. Thus, the pair-fed controls had calorie intakes identical to those of the alcohol-fed rats but did not receive alcohol.

After 24 days of ethanol treatment, rat chow was withheld for 5 days while ethanol administration continued in order to stimulate an alcoholic binge.

After the fasting period, rats were sacrificed by decapitation. Muscles were then excised and homogenates were prepared (for details, please see Chapter 2).

3.2.3 Biochemical Assays of the Muscle Homogenates

Details of the various assays have been described in Chapter 2.

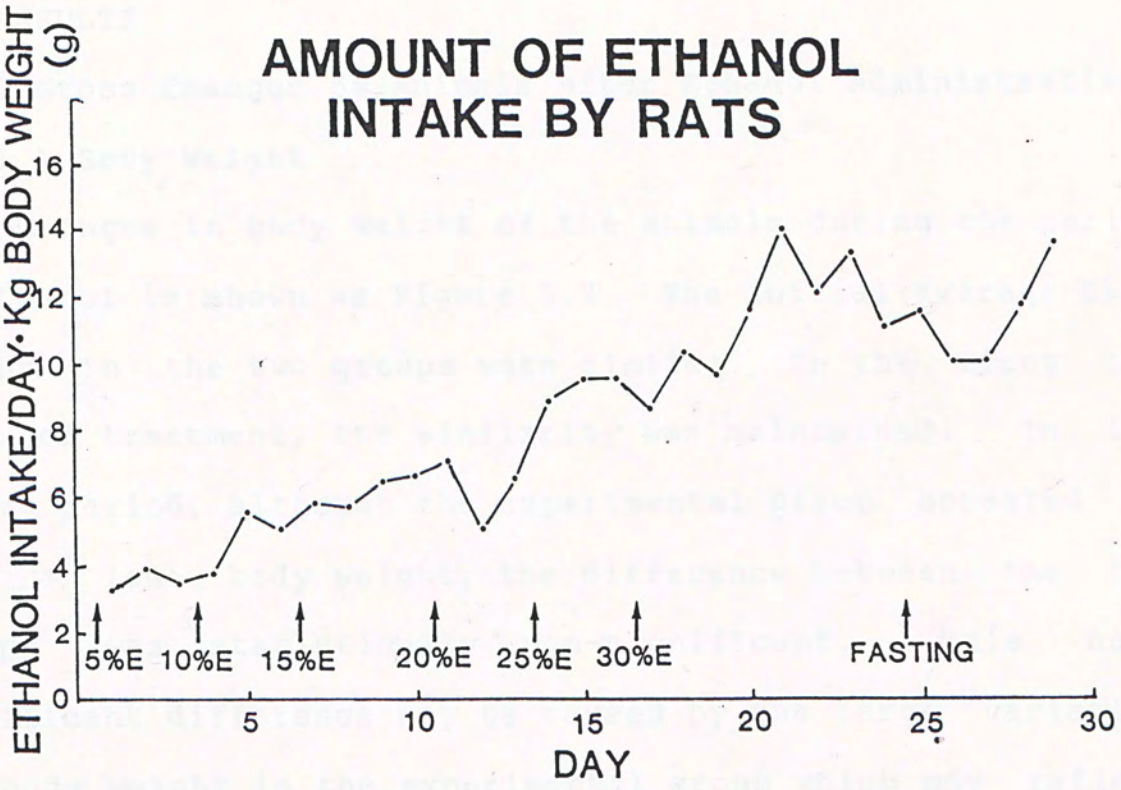

Figure 3.1 Amount of ethanol intake by rats during the period of treatment. Each change in the amount of alcohol given is indicated by a arrow. E stands for ethanol solution.

3.2.4 Statistical Methods

Values have been presented as means ± standard error of the mean (s.e.m.). The significance of differences between mean values was assessed by Student's two-tailed t-test with a probability level of 5 per cent or less.

3.3 RESULTS

3.3.1 Gross Changes of Animals After Ethanol Administration

3.3.1.1 Body Weight

Changes in body weight of the animals during the period of ethanol is shown as Figure 3.2. The initial average body weight in the two groups were similar. In the first ten days of treatment, the similarity was maintained. In the later period, although the experimental group appeared to have a lower body weight, the difference between the two groups was statistically non-significant. This non-significant difference may be caused by the large variation of body weight in the experimental group which may reflect the different susceptibility of individual animals to the treatment. However, significant difference in body weight appeared in the last few day of treatment when the rats were deprived of food. The weight loss in the experimental animals averaged 16 percent of the initial weight after 24 days of alcohol treatment while the pair-fed control animals lost only 7% of body weight in this period. This is comparable to the result of Haller's group (Haller and Drachman, 1980). After an additional 5 days of fast, the

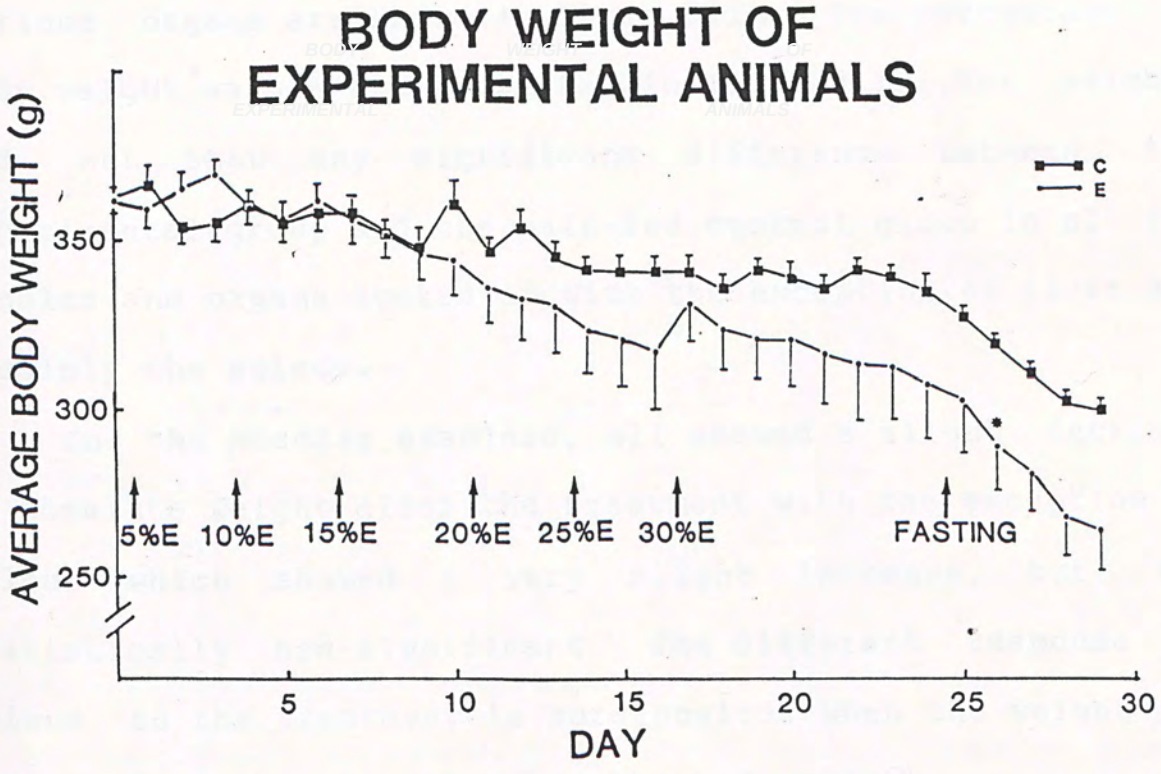

Figure 3.2 Body weight of ethanol treated (E) and pair-fed control (C) animals during the period of treatment. Every change in the amount of alcohol given is indicated by an arrow. The result are expressed as average ± S.E.M. Significant difference between the two group occurred only on the days after the "*" mark.

body weight loss of the experimental group rose to 27% while that of the control group was 18%. The difference between the two groups became significant in this period. This may reflect the fact that fasting manifested the effect of ethanol on the animals.

3.3.1.2 Organ and Muscle Weight

The weights of the muscles of the hind limbs and the various organs are shown in Table 3.1A. The percentage of body weight values are tabulated in Table 3.1B. The weights did not show any significant difference between the experimental group and the pair-fed control group in all the muscles and organs looked at with the exception of liver and possibly the soleus.

In the muscles examined, all showed a slight decrease in absolute weight after the treatment with the exception of soleus which showed a very slight increase, but all statistically non-significant. The different response of soleus to the treatment is more obvious when the weight is expressed as the percentage of the body weight.

For the other organs, liver showed significant decrease in both absolute weight and percentage of body weight. On the other hand, the spleen appeared to be increased substantially, but the large variation in the experimental group rendered the result non-significant. No significant changes in the heart and kidney were detected.

(A) Weight (g)

	Control	Experimental	% Change	P Value
Gastrocnemius	2.313 ± 0.109	2.118 ± 0.055	-8%	N.S.
TA	0.746 ± 0.021	0.673 ± 0.031	-10%	N.S.
EDL	0.228 ± 0.011	0.209 ± 0.009	-8%	N.S.
Soleus	0.178 ± 0.005	0.186 ± 0.005	+4%	N.S.
Heart	1.014 ± 0.132	1.094 ± 0.104	+8%	N.S.
Kidney	2.819 ± 0.206	2.736 ± 0.279	-3%	N.S.
Liver	9.528 ± 0.529	6.504 ± 0.660	-32%	P<0.005
Spleen	0.514 ± 0.041	0.654 ± 0.159	+27%	N.S.

(B) Percentage of body weight

	Control	Experimental	% Change	P Value
Gastrocnemius	0.770 ± 0.016	0.801 ± 0.014	+4%	N.S.
TA	0.248 ± 0.007	0.255 ± 0.003	+3%	N.S.
EDL	0.076 ± 0.004	0.079 ± 0.002	+4%	N.S.
Soleus	0.059 ± 0.002	0.071 ± 0.003	+20%	P<0.005
Heart	0.338 ± 0.044	0.414 ± 0.034	+22%	N.S.
Kidney	0.939 ± 0.069	1.044 ± 0.117	+11%	N.S.
Liver	3.169 ± 0.167	2.457 ± 0.203	-22%	P<0.025
Spleen	0.170 ± 0.013	0.241 ± 0.055	+42%	N.S.

Table 3.1 Weight (A) and percentage (B) of body weight of the ethanol treated (Experimental) and pair-fed control (Control) animals. "% change" is the percentage change of these parameters in the experimental group as compared to the control group.

3.3.2 Changes in Enzyme Activities in Muscles

3.3.2.1 Proteolytic Enzymes

The activity of total acid proteinase was assayed in the muscle homogenates. The results are shown in Figure 3.3. Only gastrocnemius gave a significant change in the total activity and a significant sizable change in the specific activity. The changes were 10% and 24% in the specific and total activities, respectively.

Since acidic proteolytic activities are mainly contributed by lysosome, the activities of individual enzymes were determined.

The activity of a lysosomal proteinase, cathepsin B, in muscle homogenates of the treated and control animals was determined and the results are shown as Figure 3.4. For this enzyme, it was again gastrocnemius which gave significant change in the total activity but this time it was a decrease. The specific activity in the experimental group was also lowered but the extent was much less and the difference to the control was not significant statistically.

The activity of another lysosomal enzyme, acid phosphatase, is shown in Figure 3.5. For total activity of gastrocnemius and EDL, a result similar to that of cathepsin B was obtained. The total activity decreases significantly in gastrocnemius and slightly in EDL upon ethanol treatment. On the other hand, the specific activity in these two muscles increases slightly. In soleus, specific activity

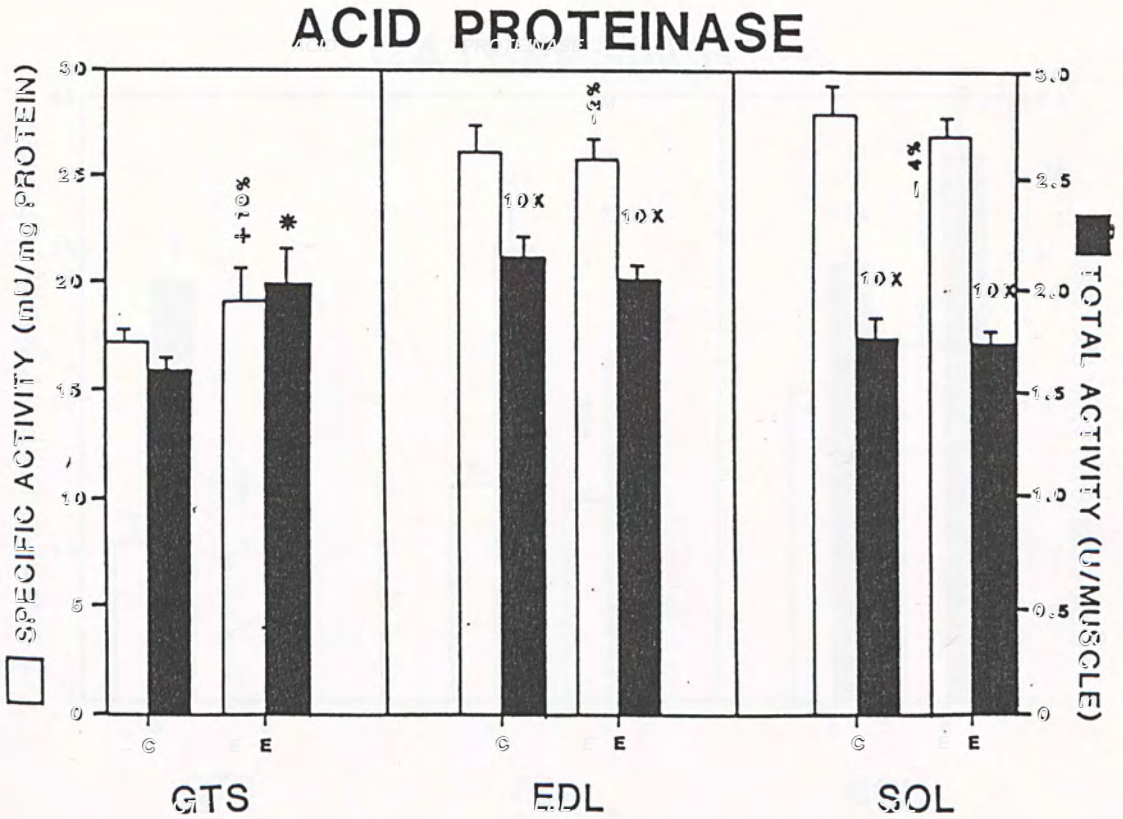

Figure 3.3 Acid proteinase activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : $P<0.05$ as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

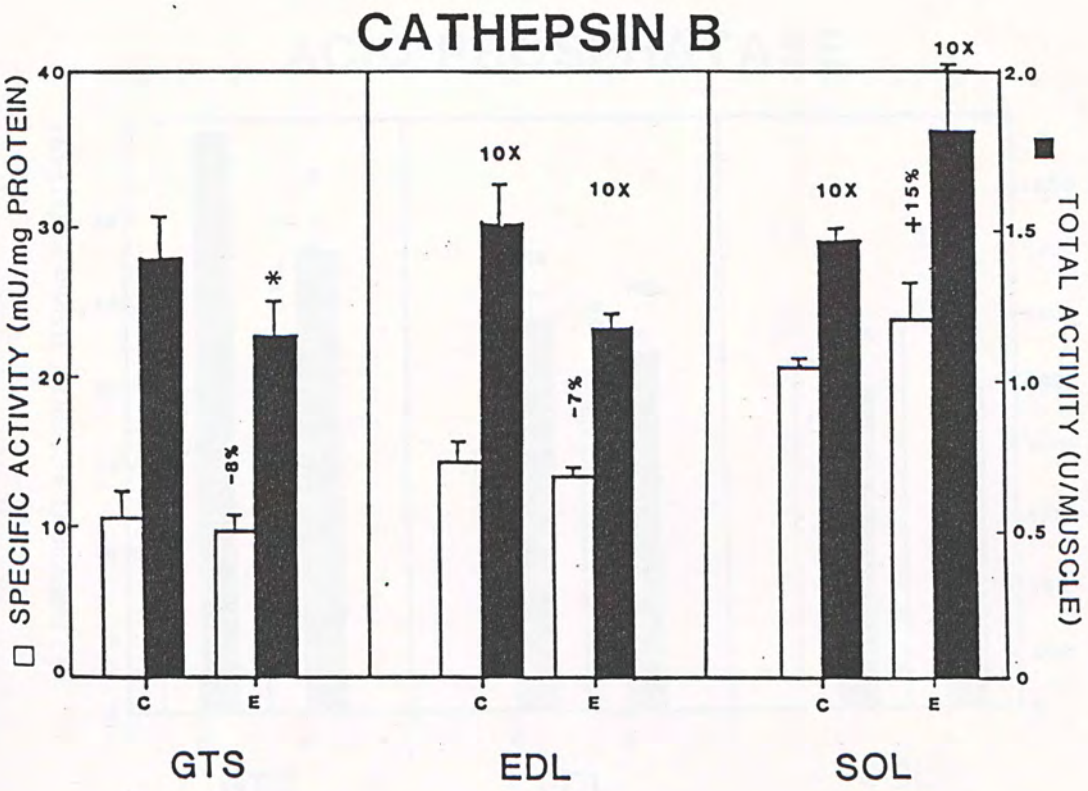

Figure 3.4 Cathepsin B activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : $P<0.05$ as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

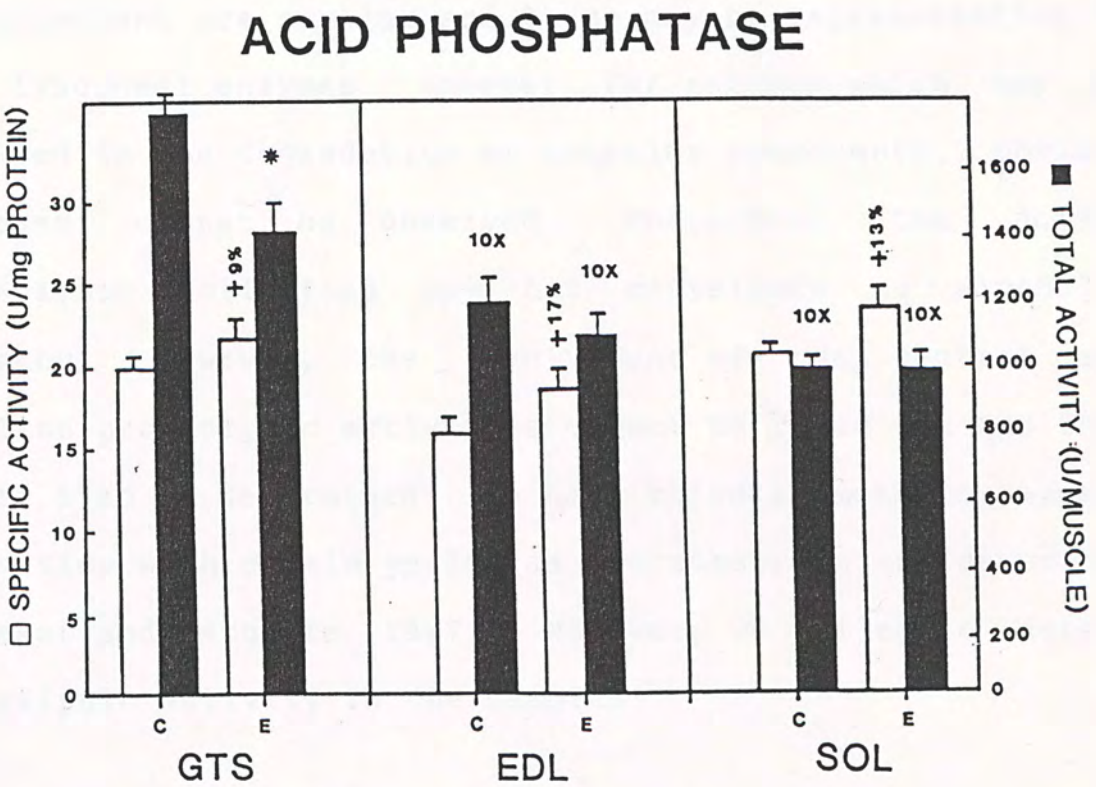

Figure 3.5 Acid phosphatase activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : $P<0.025$ as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

elevates by 13%, similar to the situation of cathepsin B. The total activity in soleus does not change, like that of acid proteinase.

From these results, it appears that the patterns of changes in cathepsin B and acid phosphatase in response to the treatment are similar and these may be representative of the lysosomal enzymes. However, for enzymes which may be involved in the degradation of cellular components, obvious increase cannot be observed. Therefore, the acidic proteolytic activities may not contribute to alcoholic myopathy. However, the involvement of the neutral and alkaline proteolytic activities cannot be ruled out and they should also be determined. We have tried to measure calpain activities with casein yellow as the substrate as described by Baker and Margolis (1987). However, we failed to detect any calpain activity in the samples.

3.3.2.2 Free Radical Scavenging Enzymes

Cytochrome oxidase, superoxide dismutase (SOD), catalase and GSH-peroxidase form the enzyme system to scavenge the free radicals in cells. Only the activities of catalase and SOD were assayed in this study.

The catalase activity in the muscle homogenates was shown in Figure 3.6. In gastrocnemius and EDL, elevation in activity was observed. Specific activity and total activity in gastrocnemius increased by 139% and 95%, respectively. In EDL, only the specific activity increased significantly. In soleus, no significant change was observed. This

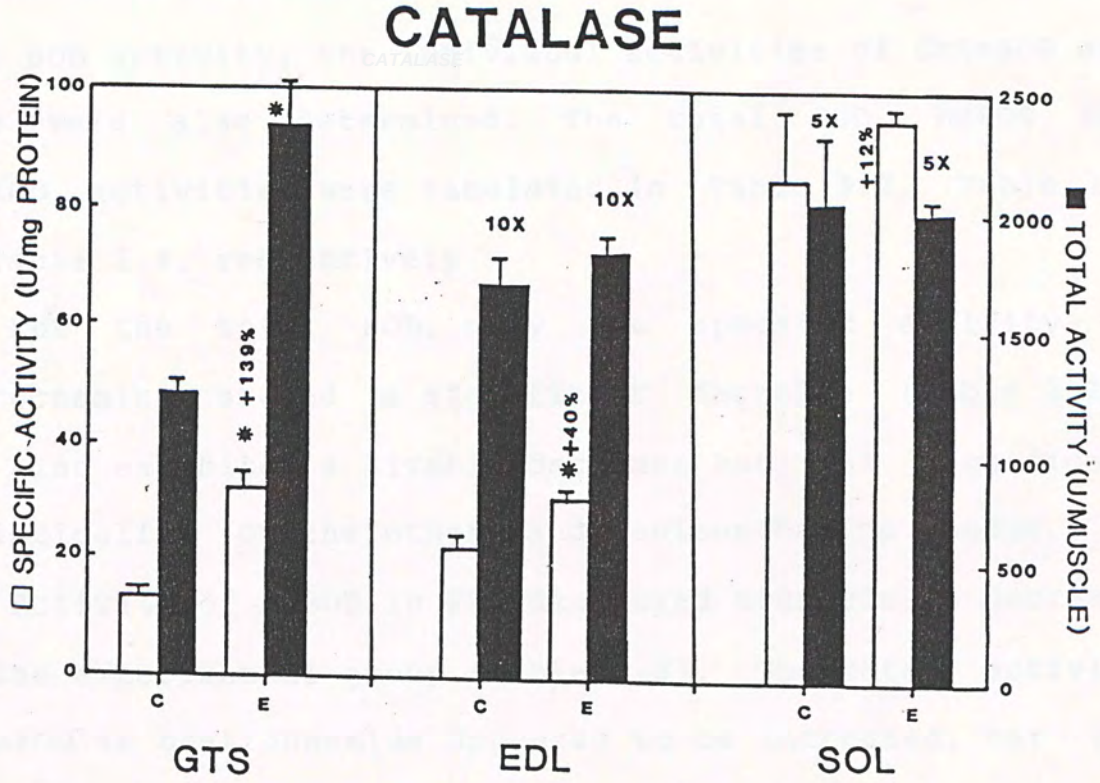

Figure 3.6 Catalase activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : $P<0.005$ as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

demonstrated once again that soleus respond to ethanol treatment in a manner as compared to the other muscles examined.

In eukaryotes, SOD generally composes of both CuZnSOD and MnSOD (Fridovich, 1978). For this reason, besides the total SOD activity, the individual activities of CuZnSOD and MnSOD were also determined. The total SOD, MnSOD and CuZnSOD activities were tabulated in Table 3.2, Table 3.3 and Table 3.4, respectively.

For the total SOD, only the specific activity in gastrocnemius showed a significant decrease (Table 3.2). EDL also exhibited a sizable decrease but not significant statistically. On the other hand, soleus had no change.

Activity of MnSOD in EDL displayed significant decrease in the experimental group (Table 3.3). The total activity of MnSOD in gastrocnemius appeared to be increased, but the increase was not significant statistically. Again, soleus did not show any alteration in MnSOD activity.

CuZnSOD activity did not change significantly in any of the muscles examined (Table 3.4).

Taken together, it seems that the free radical scavenging enzyme activities in soleus do not alter upon ethanol treatment. The change is most obvious in gastrocnemius in which both the specific activities of catalase and total SOD displayed significant changes, though the direction of the changes of these two enzymes was opposite. A similar relation was also found in EDL between catalase and MnSOD activities.

Specific Activity

	Control	Experimental	% Change	P Value
Gastrocnemius	32.11 ± 1.60	27.83 ± 1.73	-13%	P<0.005
E.D.L.	33.37 ± 2.94	31.05 ± 3.04	-7%	N.S.
Soleus	37.75 ± 0.61	38.24 ± 4.20	+1%	N.S.

(Unit : U / mg Protein)

Total Activity

	Control	Experimental	% Change	P Value
Gastrocnemius	2989 ± 150	2917 ± 208	-2%	N.S.
E.D.L.	270.6 ± 23.9	241.6 ± 23.6	-11%	N.S.
Soleus	236.0 ± 3.8	245.4 ± 26.9	+4%	N.S.

(Unit : U / Muscle)

Table 3.2 Total SOD activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) rats. Values are expressed as average ± S.E.M. "% change" is the percentage change of these parameters in the experimental group as compared to those in the control group.

Specific Activity

	Control	Experimental	% Change	P Value
Gastrocnemius	6.26 ± 0.12	6.16 ± 0.31	-2%	N.S.
E.D.L.	6.69 ± 0.09	6.01 ± 0.39	-10%	P<0.05
Soleus	6.36 ± 0.51	6.32 ± 0.37	-1%	N.S.

(Unit : U / mg Protein)

Total Activity

	Control	Experimental	% Change	P Value
Gastrocnemius	584.3 ± 20.6	646.6 ± 41.7	+11%	N.S.
E.D.L.	54.25 ± 0.76	46.83 ± 3.07	-14%	P<0.025
Soleus	39.78 ± 3.16	40.53 ± 2.37	+2%	N.S.

(Unit : U / Muscle)

Table 3.3 MnSOD activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) rats. Values are expressed as average ± S.E.M. "% change" is the percentage change of these parameters in the experimental group as compared to those in the control group. Specific activity is the total enzyme units relative to the total amount of all non-collagen proteins present. Total activity is the total units of the enzymes in a muscle.

Specific Activity

	Control	Experimental	% Change	P Value
Gastrocnemius	25.85 ± 1.61	21.67 ± 1.44	-16%	N.S.
E.D.L.	26.68 ± 3.01	25.00 ± 3.09	-6%	N.S.
Soleus	31.38 ± 0.62	31.93 ± 4.56	+2%	N.S.

(Unit : U / mg Protein)

Total Activity

	Control	Experimental	% Change	P Value
Gastrocnemius	2405 ± 149	2270 ± 168	-6%	N.S.
E.D.L.	216.3 ± 24.4	194.8 ± 24.1	-10%	N.S.
Soleus	196.2 ± 3.8	204.9 ± 29.3	+4%	N.S.

(Unit : U / Muscle)

Table 3.4 CuZnSOD activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) rats. Values are expressed as average ± S.E.M. "% change" is the percentage change of these parameters in the experimental group as compared to those in the control group. Specific activity is the total enzyme units relative to the total amount of all non-collagen proteins present. Total activity is the total units of the enzymes in a muscle.

3.3.2.3 Energy Metabolizing Enzymes

In this study, only the carbohydrate metabolizing enzymes were investigated. Malate dehydrogenase (MDH) was chosen as a representative of the enzymes in the citric acid cycle in oxidative metabolism. The MDH results were shown in Figure 3.7. For specific activity, only gastrocnemius displayed a significant change in the experimental group, i.e. an increase of 16%. In EDL and less so in soleus, lower total activities of MDH in the experimental group were detected. In this aspect, it seems that gastrocnemius respond to the treatment in a different manner as compared to the other two muscles.

Lactate dehydrogenase (LDH), the enzyme involved in the final step of anaerobic glycolysis of carbohydrate was assayed and the results shown in Figure 3.8. The specific activity altered significantly only in soleus, an increase of 38%. In contrast, significant decreases were found in both gastrocnemius and EDL in total activity.

Hexokinase catalyses the phosphorylation of glucose and is important as a first step in glycolysis and the pentose shunt. Hexokinase was assayed and the results were shown in Figure 3.9. Only in gastrocnemius a significant decrease was detected. The activity of this enzyme appeared to be decreased in EDL but the difference was not significant statistically. For soleus, the specific activity increased slightly and the total activity had no change.

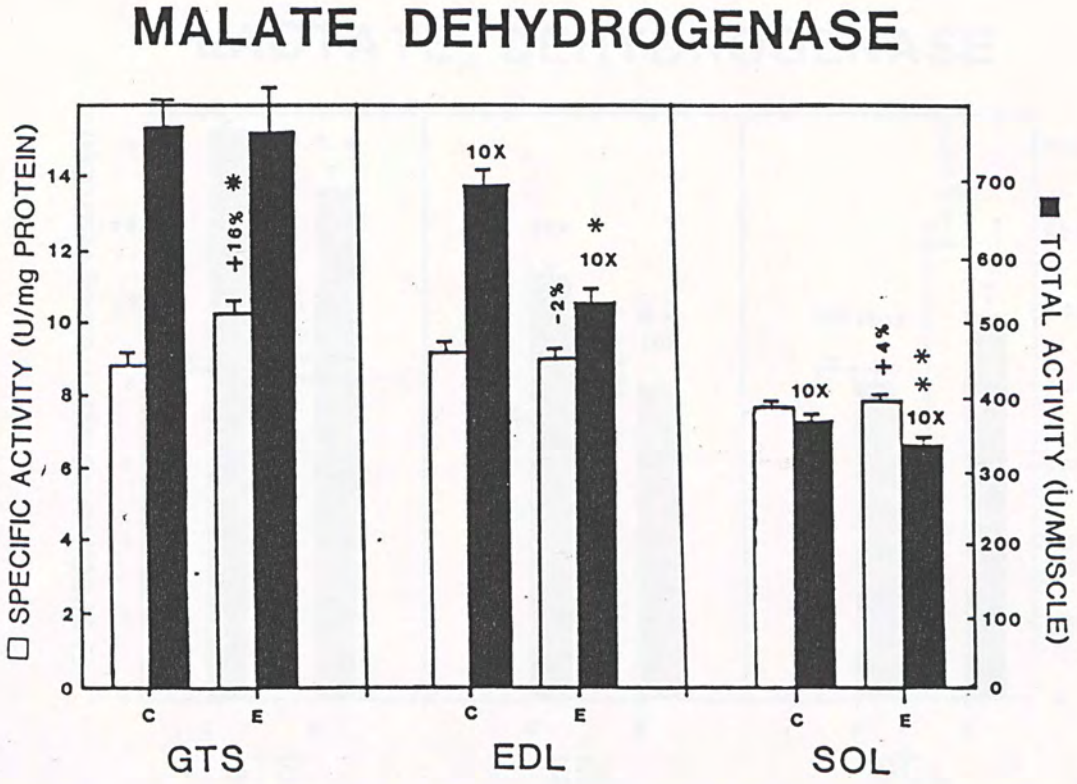

Figure 3.7 Malate dehydrogenase activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : P<0.01; : P<0.025; : P<0.05 as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

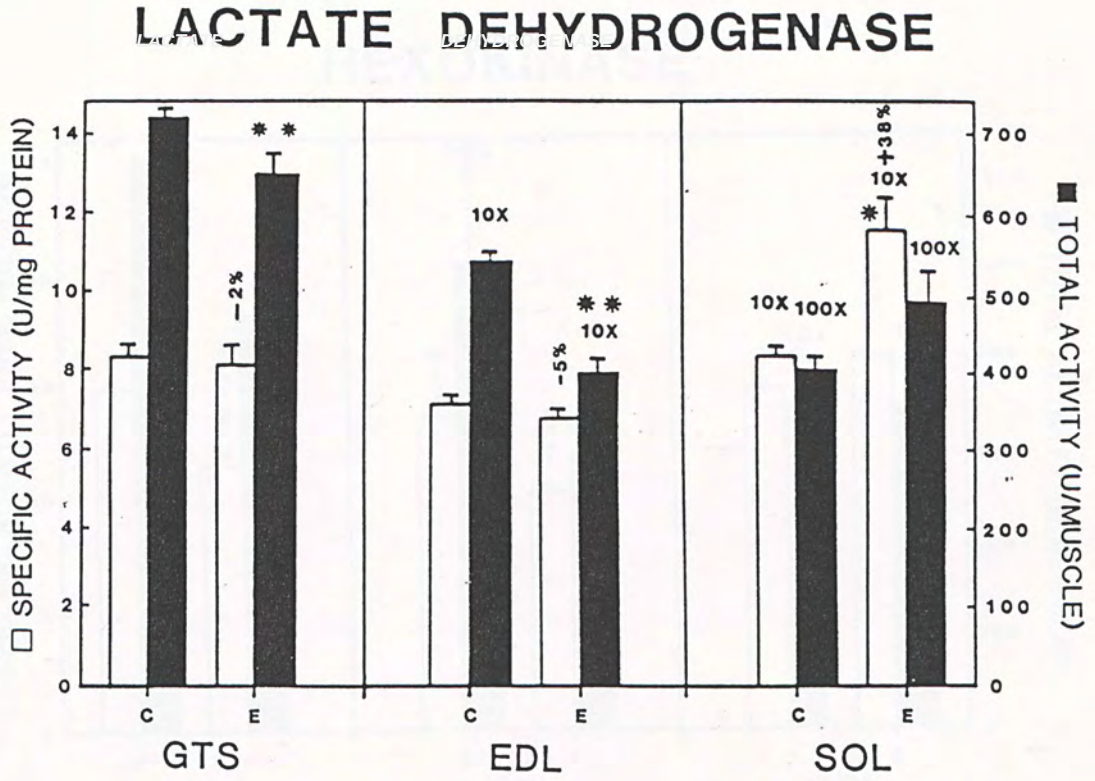

Figure 3.8 Lactate dehydrogenase activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : $P<0.05$; ** : $P<0.025$ as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

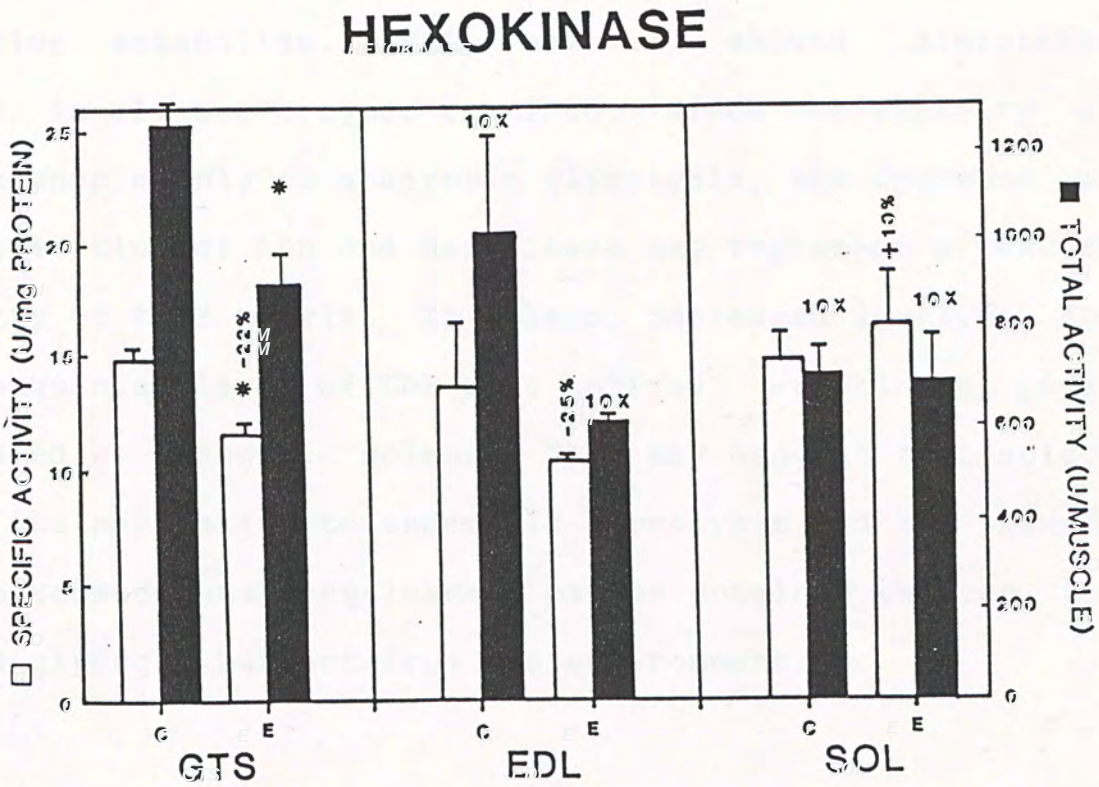

Figure 3.9 Hexokinase activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : P<0.005 as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

To summarize the results on the energy metabolizing enzymes, it was found that gastrocnemius exhibited elevated MDH level but reduced level of LDH and hexokinase upon ethanol treatment. This reflected that gastrocnemius responds to the treatment by becoming more dependent on oxidative metabolism. EDL, however, showed diminished levels in all the enzymes examined. Since the activity of EDL depends mainly on anaerobic glycolysis, the decrease in the activities of LDH and hexokinase may represent a reduced activity of this muscle. In soleus, decreased level of MDH and augmented level of LDH were noticed. Hexokinase Level displayed no change in soleus. This may suggest that soleus shift its metabolism to anaerobic glycolysis and the source of increased sugar requirement may be obtained through the stored glycogen but not from the environment.

3.3.2.4 Other Enzymes

Alkaline phosphatase activity was reported to be used as an indicator of sarcoplasmic reticulum and endoplasmic reticulum membrane integrity (Davies et al., 1982). It was also reported that only a low alkaline phosphatase activity existed in normal muscles and this activity would increase in severely atrophying muscle (McComb et al., 1979). For this reason, activity of alkaline phosphatase was assayed and the results shown in Figure 3.10.

Only in soleus a significant increase was detected. The specific activity in gastrocnemius also increase by a certain extent but the total activity decrease slightly. In

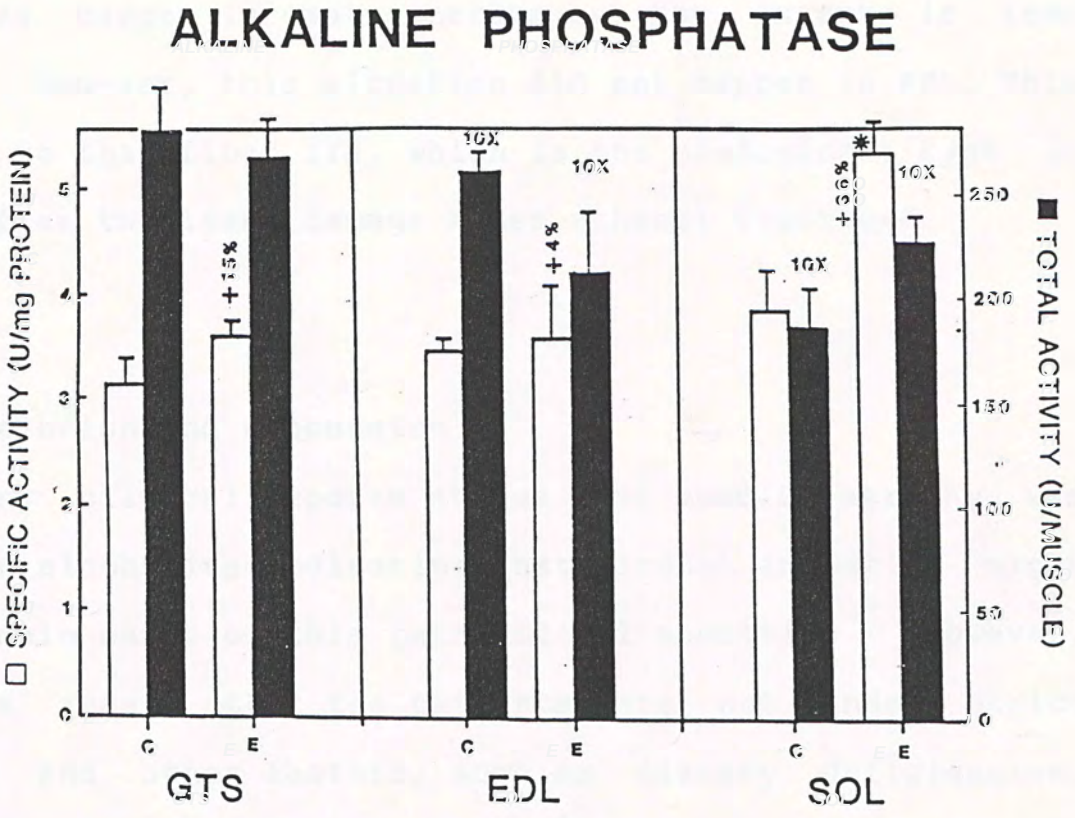

Figure 3.10 Alkaline phosphatase activity in muscle homogenates of ethanol treated (E) and pair-fed control (C) animals. The percentage value is the percentage change of specific activity in the experimental group as compared to the control group. GTS : gastrocnemius; EDL : extensor digitorum longus; SOL : soleus. * : $P<0.05$ as compared to the control group. 10X stands for that the value shown here is ten fold of the actual value. Specific activity is the total enzyme units relative to the total amount of non-collagen proteins present. Total activity is the total enzyme units in a muscle.

EDL, no change in specific activity was found while the total activity decrease to a degree. These results may suggest that soleus loses its membrane integrity and is undergoing atrophy after the treatment. The same situation may also happen in gastrocnemius but the extent is less severe. However, this situation did not happen in EDL This may due to that fiber IIa, which is the predominant type in EDL, suffer the least damage after ethanol treatment.

3.4 Conclusion and Discussion

Many clinical reports stated that muscle atrophy was found in alcoholics indicating that alcohol ingestion might be the main cause of this pathological condition. However, for the reason that the patients were not under strict control and other factors, such as dietary deficiencies, which accompanied alcohol abuse, it has been difficult to attribute the observed muscle atrophy to alcohol ingestion alone.

Song and Rubin (1972) tried to reproduce the alcoholic effect on skeletal muscle by administrating alcohol to well-controlled human volunteers. They found that chronic ingestion of ethanol for 28 days, independent of nutritional or other factors, leads to increased serum creatine kinase (CK) activity and striking ultrastructural changes in skeletal muscle. However, they also found that clinical evidence of muscular disease was noted only in a minority of

tested chronic alcoholics and the increase in serum CK activity appeared to be variable. Although the model was not entirely successful, it gives support to the notion that ethanol itself, without the contribution of other factors, could give rise to the muscle atrophy reported.

An animal model for acute alcoholic myopathy was developed in rats by Haller and Drachman (1980). They had also illustrated that the histological features of the experimental animals closely parallel the findings in various clinical reports about the disease in human patients (Haller, 1985). The model provides the opportunity to look into the pathology of the disease and its biochemical basis.

Concerning the gross changes of animals in this model, only weight loss was reported by Haller's group (Haller and Drachman, 1980). They noted that weight loss in the experimental animals averaged 16 % of the initial weight after 3 weeks of alcoholic exposure (rats were kept in chamber filled with ethanol vapor and giving blood alcohol concentrations in the range of 100 to 300 mg per 100 ml). We found that the experimental rats lost 16% of weight after 24 days of ethanol treatment. The two results were extremely comparable.

It was also found that although the caloric intake was the same in both the ethanol treated group and the pair-fed control group, the ethanol treated group loses its body weight by a greater extent, suggesting that alcohol is exerting some effects on the whole body of the animals. Also, more drastic weight loss appeared in the fast period,

particularly for the ethanol treated group. It has been shown that food intake before alcohol ingestion can lower the peak blood alcohol concentration, and reduces the time required to clear alcohol from the blood (Sharma and Moskowitz, 1978). Therefore the drastic effect of ethanol ingestion on the body weight in the fast period may be caused by toxicity of alcohol which concentration was maintained higher and for a longer time in the blood in the absence of food intake.

Absolute and the percentage to the body of liver in the ethanol treated rats decreased significantly. It is no doubt that liver is the most susceptible organ to the influence of alcohol for its role in the metabolism of alcohol. The decreased weight may indicate reduction in some components of the liver. This is in fact contrary to the situation reported by Lieber (1980) who showed an increase in liver weight after ingestion of ethanol. Fat deposition and liver enlargement were reported. It was shown that lipids accounted for half the increase in the liver dry weight (Lieber et al., 1965), while the other half is almost totally accounted for by an increase in proteins (Baraona et al., 1975). But these effects appeared in the cases of ethanol ingestion only. The weight decrease reported in this study may be the result of the combination of ethanol treatment and fasting.

It is clear that ethanol has a differential effect on hepatic protein synthesis (Rothschild et al., 1987). A

stimulus to collagen synthesis occurs simultaneously with a depression of albumin and transferrin synthesis and release. On the other hand, it was reported that decreased intake of dietary protein or decreased absorption of amino acids derived from dietary protein contribute to the depletion of amino acids in the liver (Lieber, 1980). Therefore, food deprivation depletes amino acid supply in liver and thus limits the extent of protein synthesis but on the other hand the depression effect maintains. As a result, the protein content decreases in liver and therefore giving a lower weight after the treatment.

The heart in our experimental animals displayed certain increase in its absolute weight and percentage of the body weight. This agrees to the findings of various clinical reports which described cardiac dilation and hypertrophy secondary to chronic alcoholism (Bing and Tillmanns, 1977). As a result, heart diminished its contractility.

The few autopsy studies on acute alcoholic myopathy have indicated that ethanol exerts an effect on most skeletal muscles, including pharyngeal muscles, the strap muscles of the neck, and the diaphragm (Hed et al., 1962; Kahn and Meyer, 1970). However, for the muscles investigated in this study, only soleus showed significant change in percentage in the experimental group. The different response of soleus to the treatment agrees to the finding that the severity of myopathy within sampled muscles varied according to the proportion of type I fibers contained in the muscles (Haller, 1985). So, the effect of

alcohol may be reflected in the weight of soleus in which type I fiber predominates (Haller, 1985; Squire; 1986).

The weight increase may stem from the higher capacity of water retention in this muscle after the treatment. This is indicated by the result of protein concentration determination in the muscle homogenates. The protein concentration of homogenate of ethanol treated soleus homogenate was 16% lower than that of the control. On the other hand, the increase of weight may be contributed by an elevation in fat deposition in the muscle (Song and Rubin, 1972). However, in our preliminary test of the lipid content in the whole homogenate, little decrease was in fact observed.

Another possible cause for the weight increase is the increase in connective tissue, especially the deposition of its main component, collagen, which had been shown in the liver of ethanol-fed animals (Lieber, 1980).

For the other muscles examined, TA was reported to be composed of about half of type I and half of type II fibers (Close, 1972). EDL was found to be made up of mainly type II (IIb) fibers (Close, 1972; Haller, 1985). Medial head of gastrocnemius was shown to be a fast-contracting muscle which contains a higher proportion of type IIa or type IIb fibers (Bowman, 1980; Simard et al., 1985). In addition, it was reported that in gastrocnemius most type II fibers are IIb (Brook and Kaiser, 1970). The reason why these muscles do not display significant response to the ethanol treatment

may be due to the fact that they are not made up of predominary type I fiber.

Electronmicroscopic examination of muscle biopsy from patients with acute attack showed that myofibrils were disordered, entire cells were swollen and a disarray of organelles was noted (Geller and Rubin, 1977). Since the order of myofibrils in a muscle is maintained by the integrity of M-line and Z-line, the disorder of myofibrils may imply the elevation in muscle proteolytic activities acting on the myofibrillar proteins. Therefore, it is valuable to find out what proteolytic activities are involved in the atrophy. This may also be helpful in probing the cause of the increased protein degradation. In skeletal muscle, the proteolytic activities described so far are the lysosomal proteinases, the cytosolic proteinases such as calpain, and some alkaline serine proteinase originated from invading mast cells. The activities of lysosomal proteinases and the acid proteinases were investigated in the present study.

Lysosomal proteinases activities were found to increase in various conditions of muscle atrophy, such as muscular dystrophy (Katunuma and Noda, 1980), denervation (McLaughlin et al., 1974), toxin-induced paralysis (Tågerud et al., 1986). For a long time, lysosomes were not able to be detected in normal muscle by light or electron microscopy. It was even suggested that the usual lysosomal enzymes associated with Golgi membranes or the sarcoplasmic reticulum in muscle are not involved in the acceleration of

catabolism of muscle protein (Maskrey et al., 1977). However, it had been reported later that cathepsins B and D were visualized in muscle cells lysosomes by electron microscopy (Bird et al., 1980). This implies that the assessment of activities of lysosomal enzymes may reflect the involvement of lysosome in muscles.

In our study, acid proteinase activity showed significant increase only in the total activity of gastrocnemius. Activity in EDL and soleus showed almost no change. On the other hand, total activity of cathepsin B decreased significantly in gastrocnemius only. The opposite effect of these two enzymes in gastrocnemius implied the possibility that there is a few types of lysosome which have different cellular origins (Bird et al., 1980) and that the proteinase content of these lysosomes are different. Lysosomes containing acid proteinases other than cathepsin B may increase in the gastrocnemius of the ethanol treated rats while those containing cathepsin B decrease in the same situation. Activity of cathepsin B in EDL also decrease. The most striking different result about cathepsin B was noted in soleus which showed increase in both specific activity and total activity. Combining with the finding of Haller (1985) that only the muscles containing mainly type I fibers may undergo atrophy, cathepsin B activity may correspond to the wasting of the muscle. However, the increase in this activity was not significant and it is likely that other proteolytic activities are involved in the

atrophy.

In gastrocnemius, acid proteinase activity increase while cathepsin B activity decrease. This showed that our method of acid proteinase assay did not reflect the situation of cathepsin B. Since the pH value in acid proteinase assay was 3.5, it is possible that other proteinases having optimal pH range about 3.5 increase after the treatment. The possible proteinases are cathepsin D, cathepsin E, or 'collagenolytic cathepsin' (Barrett, 1977).

Acid proteinase activity increase only in gastrocnemius but not in both EDL and soleus. Because that type IIb fiber exist only in a little amount in EDL and soleus (please refer to Chapter 2), it is quite possible that the increase in the enzyme activity was contributed by type IIb fibers in gastrocnemius.

The pattern of acid phosphatase was somewhat similar to that of cathepsin B. In the report of Haller (1985), increase in acid phosphatase staining in degenerating fibers and in the interstitium surrounding degenerating cells represented lysosomal activity in necrotic cells and phagocytes. Also, it was reported that acid phosphatase activity increase in atrophied fibers where muscle fiber were replaced by connective tissue and suggesting that the high level of enzyme activity was contributed by the invading cells (Beckett and Bourne, 1957). Thus, it is not clear whether the change in lysosomal activity observed represent that in the necrotic cells or that in the phagocytes or other invading cells. As no significant

result was found in soleus, the most susceptible muscles proposed, it is quite possible that other proteinases would be involved in the wasting process.

Corresponding to the electronmicroscopic observation that myofibrils were disordered (Geller and Rubin, 1977), it is quite possible that calpain which had been reported to remove the Z-line from myofibrils in the present of Ca^{2+} at neutral pH was involved in the atrophy. It is because that removal of Z-line will result in the disassembly of the array of myofibrils. We had tried to assay calpain activity in the muscle homogenates but failed.

Another observation in the electronmicroscopic examination of the atrophying muscles was dilation of endoplasmic reticulum (Geller and Rubin, 1977). It was suspected that endoplasmic reticulum lost its integrity in the degenerated muscles. Alkaline phosphatase activity was reported to be used as an indicator of sarcoplasmic reticulum and endoplasmic reticulum membrane integrity (Davies et al., 1982). It was also reported that alkaline phosphatase activity was limited to the capillaries and the intima of larger vessels in normal muscles and in a small amount. This activity would increase in severely atrophying muscles and appear in the atrophying muscle fibers and in the very fine connective tissue fibers surrounding them, (Beckett and Bourne, 1957; McComb et al., 1979). Activity of alkaline phosphatase in the ethanol treated muscles was examined. The result showed that significant elevated level

of alkaline phosphatase happened only in specific activity of soleus. The increase activity in soleus illustrated that only soleus underwent severe wasting. Also, it also agreed with the assumption that the membrane of some organelles lost its integrity.

A significant source of membrane damage may be the peroxidation of the lipid components of the membrane. In liver, lipoperoxidation has been shown to be increased following acute and chronic alcohol ingestion (Videla et al., 1980). Also, fasting induced a greater reduction in liver GSH content after the ingestion of ethanol. It was also shown that content of GSH showed an inverse relation to the production of lipid peroxide (Videla et al., 1980). So, fasting increase the production of lipid peroxide which may correspond to the damage in liver. The similarity between the condition of producing higher degree of lipid peroxidation in liver and the treatment we used raise the question whether or not lipid peroxidation occur in muscle. For the limitation of the method of preparation of muscle homogenate, level of lipid peroxide had not been measured. Rather, some enzymes involved in the scavenging of free radicals were assessed.

It has been reported that alcohol-induced enhancement of liver lipid peroxidation occurred concomitantly with an increase in the activity of SOD and in that of some enzymes which generate $O_2 \cdot^-$. However, no change in the activities of catalase and GSH peroxidase-reductase couple was found (Valenzuela et al., 1980). These result was explained by

the increase in the generation of active radicals for the induction of microsomes after chronic ethanol consumption (Lieber, 1980), the decrease in the antioxidant, GSH (Videla et al., 1980), and the usual level of catalase and GSH-peroxidase (Valenzuela et al., 1980). However, it was found later that lipid peroxidation potentiated by ethanol treatment in liver were not dependent on GSH depression (Shaw et al., 1981). Free radical scavenging enzyme activities have been studied in our model to see if this also occurs in the muscles.

By examining the results we obtained, patterns different from that in ethanol treated liver were observed. Only significant decrease was found in the activity of SOD. Also, activity of catalase increase in all the samples examined. In the activity of catalase, CuZnSOD and total SOD, soleus showed a different picture to the other muscles. Contrary to the other muscles, soleus in the ethanol treated group demonstrated almost no change in catalase activity, which increase in other muscles. Soleus did not change its total and CuZnSOD activities but gastrocnemius and EDL showed a decrease.

If ethanol treatment also induce the increase in production of active radicals such as in liver, then the response of gastrocnemius and EDL may protect them from the damage of the free radicals. The lower activity of CuZnSOD may decrease the rate of production of the most toxic free radical species, i.e. H_2O_2 and $\cdot OH$, which originates from

the locally toxic species, $O_2 \cdot^-$. The elevation of catalase activity in these muscles may accelerate the scavenging of H_2O_2 and minimize the damage caused by H_2O_2 and $O_2 \cdot^-$ which derived from H_2O_2.

The results also suggested that the increased level of catalase did not originate from the phagocytes or mast cells which were reported to be increased in the atrophying muscle fibers (Haller, 1985). The results did not agree to the suggestion that catalase could be used as an indicator of the degree of muscle wasting proposed by Stauber et al. (1977) who found an increase in catalase activity in muscles associated with the wasting conditions of starvation, denervation and chloroquine myopathy.

Our results do not indicate whether production of free radicals was increased or lipid peroxidation occurred. Further information is necessary to make clear the role of lipid peroxidation in alcoholic myopathy. However, it is quite possible that the failure in responding to the increase of free radical induced by ethanol make soleus the victim of ethanol ingestion.

The level of these enzymes in different muscle atrophy had also been described in various reports. Our results were quite different from that of denervation which displayed alteration in free radical scavenging enzyme activities only in slow muscle (Asayama et al., 1986). This may be an evidence that the alcoholic myopathy does not involve the disorder of neural control.

Significant decrease in MnSOD activity was noted only

in EDL. Decrease in MnSOD was supposed to be secondary to decreased mitochondrial metabolic activity (Asayama et al., 1986; Burr et al., 1987). Alternatively, as MnSOD is located in the matrix of mitochondria, it was suggested that a change in activity of this enzyme would indicate the alteration in numbers of mitochondria in muscle (Mizuno, 1984). To see if activity of MnSOD reflect the situation of mitochondria, activity of mitochondrial enzyme, MDH, was determined.

For the activity of MDH in EDL, a significant and fairly large decrease in total activity occurred. Statistically significant but not quite large decrease occurred in soleus. The result of MDH was basically similar to that of MnSOD with the exception that specific activity of MDH increase to a certain extent. So the change in activity of MnSOD may actually be the consequence of the change in mitochondrial function.

Then, what would the change of MDH imply in the muscle atrophy? To answer this question, a whole view about the metabolism in the muscle should be investigated. In addition to MDH, activities of LDH, a typical glycolytic enzyme, and hexokinase, an enzyme corresponding to the uptake of glucose, were assayed.

In gastrocnemius, activities of LDH and hexokinase decrease while that of MDH increase. This may imply that gastrocnemius depend more on the oxidative metabolism of lipid after the ethanol treatment. In EDL, all enzymes examined showed decreased activity. This may indicate that

the metabolism in EDL decrease perhaps as a result of less exercise.

In soleus, activities of MDH and hexokinase decreased in a small scale and almost unchanged, respectively. However, activity of LDH elevated markedly. This illustrated that soleus had shifted its metabolism from the oxidative one to anaerobic glycolysis. Also, it seemed that the source of the substrate for the increase in glycolysis does not come from the blood but from an internal source. This agreed to the observation of Haller (1985) that loss of glycogen was found in necrotic muscle fibers. He had also proposed three possible sources of the loss of glycogen. They were the increased energy utilization in the cell, the reduced capacity for oxidative metabolism, and the influx of calcium and sodium which activate various ATPase, accelerating the hydrolysis of ATP and promoting glycogenolysis. From our result, it was believed that it was the higher energy requirement in the muscle which caused the increase use of the stored glycogen. However, it was not clear that how the change pattern of metabolism relate to the degeneration.

To compare these results with that from the denervation atrophy, different pattern can be observed. In denervated muscles, soleus showed decrease activity of all these enzymes. EDL exhibited decreased activity in LDH and MDH but elevated level in hexokinase. Gastrocnemius displayed the same pattern as EDL (Simard et al., 1985). However, the ethanol treated soleus had a much increased LDH activity and

unchanged MDH and hexokinase activity. This is also a proof that disorder of nerve control is not involved in alcoholic myopathy.

In DMD, necrotic muscles showed normal level of mitochondrial enzymes, reduced level of glycolytic enzymes and increased level of hexokinase (Ellis, 1978). The pattern was also different to that of alcoholic myopathy. This shows that no common metabolic change happened in the different type of muscle atrophy.

As a conclusion, the results obtained suggest that lysosomes are not actively involved in the degeneration process, that active free radical species may involve in the necrotic muscles causing the loss of integrity of the organelle membrane, and that the soleus shift its metabolic pattern from that of the oxidative one to glycolysis utilizing mainly the glycogen stored. Also, the data proposed that disorder of nerve control is not the cause of this type of myopathy.

Further experiments to be done may be the direct measurement of the level of lipid peroxide in the ethanol treated muscle to see if the process is actually involved in the damage. Also, the activities of proteinases other than the lysosomal ones are worth looking into to clarify the role of proteinases in the atrophy. If their roles are clarified, then inhibitors of these proteinases or antioxidants may be tested to see if any amelioration of the syndrome can be resulted.

CHAPTER 4 EFFECT OF DENERVATION AND CLENBUTEROL TREATMENT ON SKELETAL MUSCLES

4.1 Introduction

As described in Chapter 1, motor nerve exerts a trophic effect on skeletal muscle. If the nerve to a muscle is severed, the muscle will gradually waste over a period of weeks. This process is termed denervation atrophy and it is a demonstration that the muscle fibers are dependent upon the motoneurons for the maintenance of their normal structure (McComas, 1977). In this process, a series of physiological, biochemical, and morphological changes occur.

The most obvious change in the muscle following denervation is muscle atrophy which can be detected a few days after denervation and it goes on for a period of rapid progression. At the end of this period, only 20-40% of the original muscle mass remains (Sunderland and Ray, 1950). A large portion of the remaining mass is connective tissue.

Fine structure of denervated muscle fiber has also been examined with the electronmicroscope (Pellegrino and Franzini, 1963; Stonnington and Engel, 1973). During the period of atrophy the change in fiber area was matched throughout by a fall in the mean area of the myofibrils. Mitochondria in both type I and type II fibers enlarged at first in the longitudinal axis of the fiber. Subsequently these organelles shrank and formed clusters. Some mitochondria underwent frank degeneration and were included in autophagic vacuoles. Similarly the sarcoplasmic reticulum at first enlarged and then diminished. Other changes observed under the electronmicroscope included abnormalities of the Z-disc, irregularities and small

papillary projections of the sarcolemma, and focal dilatations of the transverse and sarcoplasmic tubules. Increased numbers of ribosomes were found between the myofibrils and under the surface membrane.

The change in the membranous structures, mitochondria and sarcoplasmic reticulum may be caused by membrane disorder induced by the peroxidation of the membrane lipid components. Increased catalase activity has been observed in denervated skeletal muscle (Stauber et al., 1977). Asayama et al. (1986) showed that CuZnSOD and GSH-peroxidase decreased significantly in slow muscle but not in fast muscle. On the other hand, the MnSOD activity was found to decrease in both types of muscles but greater in the slow twitch muscle. However, there is no report on the level of lipid peroxide in denervated muscles. Thus, the correlation between organelle changes and lipid peroxidation remains to be established. Nevertheless, the MnSOD activity was believed to reflect mitochondrial free radical production and in turn a function of mitochondrial metabolic activity (Asayama et al., 1986).

It was reported that in denervated rat calf muscles there was a rapid decrease in the high enzyme activities typical of a particular type of fiber, such as glycolytic enzymes in fast twitch muscles and citric acid cycle enzymes in slow twitch muscles (Hogan et al., 1965; Romanul and Hogan, 1965). As a result fiber types could no longer be distinguished. Since the different types of fibers were

typical in their contraction properties and mainly the metabolic pathway they adopted, the examination in energy metabolizing enzymes in denervated fibers will give information about the nerve-controlled phenotypic expression of different skeletal muscle fiber types. Results of Simard et al. (1985) showed that EDL and gastrocnemius seem to be more oxidative after denervation. Shackelford and Lebherz (1981) showed that neural information, or its consequences, is required to maintain the levels and the rates of synthesis of glycolytic enzymes in mature fast-twitch muscle fibers. They suggested that denervation results in a partial 'dedifferentiation' of these fibers. Histochemical study showed that denervation increased type II fibers in rat soleus muscle (Jaweed et al., 1955) and produced an exclusive atrophy of type II fibers in rat EDL muscle (Niederle and Mayar, 1978). Thus, the proportions of histochemical fiber types also indicated a dedifferentiation of muscle types after denervation.

By comparing the effects of denervation and disuse on the rates of oxidation and on activities of mitochondrial enzymes, Nemeth et al. (1980) suggested that nerve regulate oxidative processes in type I fiber by maintaining their mitochondrial enzymes, a mechanism different from that in type II fiber.

On the other hand, the change in activities of energy metabolizing enzymes also reflects the shift of energy source in the denervated muscles. In denervated slow and fast muscles, enzymes for anaerobic glycolysis, lactate

fermentation, citric acid cycle and ß-oxidation had a decreased activity (Simard et al., 1985). While in sciatectomized muscles, a 5-fold elevation in alanine and aspartate aminotransferases was reported (Asotra and Asotra, 1984). This revealed that increased utilization of amino acids function as a compensatory metabolic support during denervation atrophy.

The source of the amino acids for the increased utilization may be the degradation of structural proteins in the denervated muscles. Goldspink (1976) revealed that after denervation protein breakdown was increased in both muscle types when compared with internal controls. This happened 24 hours after denervation and maintained at least 10 days thereafter.

The increased protein breakdown is possibly caused by an increase in proteinase activities. Increases in lysosomal acidic proteinases activity (McLaughlin et al., 1974; Maskrey et al., 1977; Libelius et al., 1981) and in calpain activity (Elce et al., 1983; Hussain et al., 1987) have been reported.

Denervation of skeletal muscle also results in well defined physiological changes derived from changes in the muscle surface membrane systems. Sarcolemmal membrane changes include increased membrane resistance and sodium permeability, decreased potassium permeability and resting membrane potential, increased acetylcholine sensitivity, and increased synapse-forming potential (Smith and Appel, 1977).

Maltin et al. (1986b) reported that dietary administration of clenbuterol, a β_2 selective adrenoceptor agonist growth promoter, inhibits and reverses denervation-induced atrophy in rat soleus. This raises the possibility that in patients with diseases which are caused by the destruction of motoneurons or by lesion of the peripheral nerves this drug can be used to retard the atrophy process until muscles are reinnervated. It is better if the effect of this drug is specific to the denervated muscle and does not cause changes in other components of the body. Later, by measuring the fractional rate of protein synthesis and muscle protein and RNA contents, Maltin et al. (1987b) showed that in denervated muscles only half of the protein anabolism was achieved through reduced degradation, the remainder resulted from a stimulation in rate of protein synthesis.

However, the biochemical basis of the ameliorative effect of clenbuterol has not been investigated. Also, β_2-adrenergic receptor has been reported in skeletal muscle fibers but its function is still not clearly defined (Walton and Mastaglia, 1981). Thus, the study of the interaction of adrenergic activation and denervation may provide a clue to the function of the adrenergic receptor in skeletal muscle.

In this study, the effect of denervation on lipid peroxidation and on free radical scavenging enzyme activities has been studied, in addition to the biochemical effects of clenbuterol administration on denervated muscles.

4.2 Materials and Methods

4.2.1 Materials

Materials used in the various assays have been described in Chapter 2. Clenbuterol [benzyl alcohol, 4-amino-α-(t-butylamino)methyl-3,5-dichloro] used in the treatment of rats is a HCl-salt donated by Biomedia Foscama Industria Chimico-Farmaceutica S.P.A. The drug is dissolved in normal saline for injection.

4.2.2 Animal Treatment Regimen

A number of experiments using different treatment regimens had been performed, including rats with different ages and with different periods and different amounts of clenbuterol treatment.

For adult animals, male Sprague-Dawley rats of age 60 ± 5 days with body weight ranging between 350 and 400 g were used. In each experiment, 4 groups of 10 rats with similar initial body weight were used. For young animals, immediately weaned male Sprague-Dawley rats were used. They were usually 28 ± 2 days old and weighed about 100 g. In this experiment, 4 groups of 15 rats were used.

For the four groups of rats in an experiment, two groups were denervated while the others were sham-operated. During the operation, animals were anesthetized with ether. The sciatic nerve of the two hindlimbs in the hip region was exposed. About 1 cm of the nerve was removed in the

denervated groups but not the sham-operated groups. The skin and subcutaneous tissue were then closed with clips.

One denervated group and one sham-operated group were injected intraperitoneally with clenbuterol while the other two groups were treated with saline as control. Clenbuterol and saline injection were started on the next day after the operation. In experiment to determine the time effect, adult rats were killed 4 days (acute effect) or 2 weeks (long term effect) after the operation. The dosage of clenbuterol used was 0.2 mg/kg body-weight per day. In experiment to determine the age effect, animals were killed 1 week after the operation and the clenbuterol dosage used was 0.6 mg/kg body-weight per day.

In the experiment using different doses of clenbuterol, the doses used were 0.2 mg, 0.6 mg, 1 mg and 2 mg/kg body-weight per day. The rats used were adult ones and were killed after 1 week of the operation. In another experiment, rats were not treated with clenbuterol and they were killed after different periods of the denervation : 1 week, 2 week and 4 week.

After the period of treatment stated above, rats were sacrificed by decapitation. Muscles were then excised and homogenates were prepared (see Chapter 2).

4.2.3 Biochemical Assays of Muscle Homogenates

Details of the various assays have been described in Chapter 2.

4.2.4 Statistical Method

All data are presented as means ± standard error of the mean (S.E.M.). For determination of the effects of different factors (denervation and clenbuterol treatment), data were assessed by the analysis of variance (ANOVA) for the completely randomized two-factor design. On the other hand, the significance of differences between mean values were examined by t-test (LSD method). The probability level for significance is 5 percent or less.

4.3 RESULTS

4.3.1 Gross Changes of Animals

4.3.1.1 Body Weight

Change in the body weight in the time course experiment is shown in Table 4.1. The body weight of denervated rats became smaller than the control rats. By analyzing with F test for one factor, the P value obtained was smaller than 0.05 and thus the effect of denervation on body weight was significant.

Such an effect of denervation on the body weight of the experimental animals is also shown in Table 4.2 in which rats were subjected to acute (4 days) or long-term (2 weeks) denervation. Control rats increased their body during the experimental period and such increases were not significantly affected by clenbuterol administration. Denervated

Table 4.1 Body weight of rats with hind limb denervation for different periods of time

Time	Weight (g)
Control	461.7 ± 12.6^{a}
One Week	437.5 ± 7.3^{ab}
Two Weeks	439.2 ± 5.9^{ab}
Four Weeks	424.6 ± 7.0^{b}

Values with same superscript letters are not significantly different.
Denervation of the hind limbs was achieved by removing about 1 cm of sciatic nerve at the hip region under ether anesthetization. In the control rats, only sham-operation was suffered. In the sham-operation, the nerve was exposed only.

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rats also increased their body weight, but to a much less extent. Such truncated increase in body weight appeared to be slightly restored by clenbuterol administration but the effect is not statistically significant (Table 4.2).

The inability of clenbuterol to alter body weight changes in control and denervation rats is also demonstrated in Table 4.3 in which different doses of the drug were used and in Table 4.4 for adult rats with one week treatment. For young rats, however, denervation had no effect on body weight increases and clenbuterol appeared to accelerate such increases in both control and denervated groups, but the effect is not significant statistically (Table 4.4).

Table 4.2 Body weight increase in rats with acute and long term treatment of denervation and clenbuterol administration

	Acute Effect		Long Term Effect	
NS	35.75 ± 16.5^{a}	D:F<0.01	53.57 ± 8.14^{d}	D:F<0.005
NC	30.00 ± 4.56^{ab}	C: N.S.	59.38 ± 7.59^{d}	C: N.S.
DS	1.25 ± 2.39^{c}	D*C:N.S.	33.33 ± 3.96^{e}	D*C: N.S.
DC	5.00 ± 3.54^{bc}		44.55 ± 3.90^{de}	

Values are in unit of gram.
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Denervation method has been mentioned in Table 4.1. Clenbuterol was dissolved in normal saline and was administrated intraperitoneally. In the control groups, saline instead of clenbuterol was injected.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

As a whole, it seems that denervation represses the increase in body weight of adult rats but not that of young rats. Clenbuterol treatment has no effect on both the control and denervated groups as far as body weight is concerned. The decrease in body weight increment in the adult rats may be a consequence of less food intake. It was because that after denervation the legs of the rats were hardly to afford the heavy body to attain the rat chow which was placed at the top of the cage. The young rats with a

Table 4.3 Body weight changes in normal and denervated rats treated with clenbuterol in different doses

Dose	Denervated Rats	Control Rats	P Value
0.0mg	0.00 ± 2.04^{d}	38.75 ± 3.15^{ab}	D: P<0.001
0.2mg	7.50 ± 6.61^{d}	35.00 ± 4.56^{ab}	
0.6mg	20.00 ± 2.89^{c}	31.25 ± 8.99^{b}	C: N.S.
1.0mg	5.00 ± 2.04^{d}	42.50 ± 4.33^{a}	D*C:P<0.01
2.0mg	8.33 ± 3.33^{d}	41.25 ± 4.73^{ab}	

Values are expressed in unit of gram.
Doses are in unit of mg/kg body-weight per day.
Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

Table 4.4 Body weight changes in young and adult rats with denervation and clenbuterol treatment

	Young Rats		Adult Rats	
NS	55.67 ± 16.2^{a}	D: N.S.	33.33 ± 3.54^{a}	D:P<0.001
NC	63.00 ± 22.1^{a}	C:P<0.05	34.44 ± 1.30^{a}	C: N.S.
DS	55.33 ± 4.42^{a}	D*C:N.S.	21.00 ± 4.14^{b}	D*C: N.S.
DC	65.00 ± 7.07^{a}		18.00 ± 3.09^{b}	

Values are expressed in unit of gram.
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

much lower weight and thus it was easier for them to reach the rat chow. The fact that clenbuterol cannot change the weight of the rat supposed that its effect was a localized one. To verify this assumption, the organ weight and muscle weight of rats with clenbuterol administration were examined.

4.3.1.2 Organ Weight

The fact that denervation and administration of clenbuterol in different doses have no effect on weight of liver and heart is shown in Table 4.5 in which the absolute weight is listed and in Table 4.6 in which the percentage weight is recorded. Although it was not affected by denervation, kidney weight was decreased by clenbuterol. The most significant effect was found when the dose used was 0.6 mg/kg body-weight per day. It is not surprise that denervation has no effect on organ weights since it is a localized effect. The data, however, also showed that clenbuterol exerted only localized effect on the rats. The decreased kidney weight in clenbuterol administrated rats may be a result of that clenbuterol was excreted through kidney and in this process it induced damage to kidney and lowered its weight.

The organ weights in the young rats experiment are shown in Table 4.7. For liver, the interaction effect was significant in both the absolute and percentage weight. Clenbuterol treatment reversed the significant increase in

Table 4.5 Organ weight of denervated and normal rats with treatment of clenbuterol in different doses

Liver (D : P=0.058; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	14.98 ± 0.95^{ab}	16.91 ± 1.20^{a}
0.2mg	14.67 ± 1.02^{ab}	14.05 ± 0.76^{b}
0.6mg	15.25 ± 1.49^{ab}	14.99 ± 1.27^{ab}
1.0mg	13.44 ± 0.45^{b}	17.12 ± 0.64^{a}
2.0mg	14.43 ± 0.20^{ab}	15.50 ± 0.50^{ab}

Kidney (D : N.S.; C : P<0.01; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	1.749 ± 0.028^{ab}	1.790 ± 0.073^{a}
0.2mg	1.596 ± 0.045^{bcd}	1.679 ± 0.024^{abc}
0.6mg	1.573 ± 0.053^{cd}	1.502 ± 0.067^{d}
1.0mg	1.631 ± 0.083^{abcd}	1.677 ± 0.069^{abc}
2.0mg	1.603 ± 0.049^{bcd}	1.668 ± 0.036^{abcd}

... to be continued

Table 4.5 (continued)

Heart (D : N.S.; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	1.366 ± 0.061^{c}	1.469 ± 0.085^{bc}
0.2mg	1.379 ± 0.047^{c}	1.348 ± 0.034^{c}
0.6mg	1.652 ± 0.120^{ab}	1.408 ± 0.055^{bc}
1.0mg	1.604 ± 0.077^{abc}	1.513 ± 0.054^{bc}
2.0mg	1.789 ± 0.269^{a}	1.491 ± 0.071^{bc}

Values are expressed in unit of gram.
Doses are in unit of mg/kg body-weight per day.
Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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liver weight caused by denervation both in terms of absolute weight and percentage of body weight. Clenbuterol treatment alone had no effect.

In kidney a similar effect was observed. Denervation increased both the absolute weight and percentage of body weight. Clenbuterol treatment, however, decreased kidney weight on both normal and denervated rats but with a much marked effect on the denervated one. As a result, clenbuterol restored the increased kidney weight in

Table 4.6 Percentage organ weight of denervated and normal rats treated with clenbuterol in different doses

Liver (D : P=0.060; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	3.941 ± 0.250^{ab}	4.099 ± 0.292^{ab}
0.2mg	3.924 ± 0.273^{ab}	3.650 ± 0.197^{ab}
0.6mg	3.587 ± 0.351^{b}	4.066 ± 0.343^{ab}
1.0mg	3.513 ± 0.118^{b}	4.320 ± 0.162^{a}
2.0mg	3.623 ± 0.050^{b}	3.936 ± 0.127^{ab}

Kidney (D : N.S.; C : P<0.01; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.460 ± 0.007^{a}	0.434 ± 0.018^{ab}
0.2mg	0.427 ± 0.012^{ab}	0.436 ± 0.006^{ab}
0.6mg	0.370 ± 0.012^{c}	0.407 ± 0.018^{bc}
1.0mg	0.427 ± 0.022^{ab}	0.423 ± 0.017^{ab}
2.0mg	0.403 ± 0.012^{bc}	0.424 ± 0.009^{ab}

... to be continued

Table 4.6 (continued)

Heart (D : N.S.; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.359 ± 0.016^{bc}	0.356 ± 0.021^{bc}
0.2mg	0.369 ± 0.013^{bc}	0.350 ± 0.009^{c}
0.6mg	0.389 ± 0.028^{abc}	0.382 ± 0.015^{bc}
1.0mg	0.419 ± 0.020^{ab}	0.382 ± 0.014^{bc}
2.0mg	0.449 ± 0.068^{a}	0.379 ± 0.018^{bc}

Doses are in unit of mg/kg body-weight per day.
Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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denervated animals to its control value.

As in the adult rats, both the absolute and percentage weights of heart and spleen did not alter significantly after denervation or treatment with clenbuterol.

As a whole, denervation had significant effect on the liver and kidney weight of young rats but not on adult rats. Clenbuterol appeared to restore the increased organ weights in denervated rats to their control value.

Table 4.7 Organ weight of young rats with denervation and clenbuterol treatment

L	Absolute weight (g)		Percentage of body weight	
NS	7.769 ± 0.201^{b}	D: N.S. C: N.S. D*C: P<0.05	5.012 ± 0.081^{b}	D: N.S. C: P<0.001 D*C: P<0.001
NC	7.879 ± 0.255^{b}		4.854 ± 0.155^{bc}	
DS	8.631 ± 0.325^{a}		5.556 ± 0.113^{a}	
DC	7.591 ± 0.249^{b}		4.591 ± 0.088^{c}	

K	Absolute weight (g)		Percentage of body weight	
NS	0.882 ± 0.031^{ab}	D: N.S. C: P<0.001 D*C: N.S.	0.569 ± 0.012^{b}	D: N.S. C: P<0.001 D*C: P<0.025
NC	0.816 ± 0.020^{b}		0.503 ± 0.017^{c}	
DS	0.942 ± 0.028^{a}		0.607 ± 0.007^{a}	
DC	0.815 ± 0.024^{b}		0.493 ± 0.007^{c}	

H	Absolute weight (g)		Percentage of body weight	
NS	0.687 ± 0.015^{b}	D: N.S. C: P=0.066 D*C: N.S.	0.443 ± 0.009^{a}	D: N.S. C: N.S. D*C: N.S.
NC	0.732 ± 0.019^{ab}		0.451 ± 0.010^{a}	
DS	0.718 ± 0.022^{ab}		0.463 ± 0.011^{a}	
DC	0.745 ± 0.020^{a}		0.451 ± 0.008^{a}	

... to be continued

Table 4.7 (continued)

S	Absolute weight (g)		Percentage of body weight	
NS	0.564 ± 0.020[a]	D: N.S. C: N.S. D*C: N.S.	0.364 ± 0.011[a]	D: N.S. C: N.S. D*C: N.S.
NC	0.580 ± 0.021[a]		0.357 ± 0.015[a]	
DS	0.551 ± 0.026[a]		0.355 ± 0.012[a]	
DC	0.608 ± 0.023[a]		0.368 ± 0.012[a]	

L : liver; K : kidney; H : heart; S : spleen
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

4.3.1.3 Muscle Weight

The absolute and percentage muscle weights in the experiment addressing the dose effect of clenbuterol are shown in Table 4.8 and Table 4.9, respectively. In all the muscles investigated, denervation caused a significant decrease in the absolute weight and percentage of body weight. Clenbuterol alone had no significant effect, but the drug appeared to be partially effective in restoring the muscle weight decrease inflicted by denervation.

In the experiment in which the time effect was inves-

Table 4.8 Muscle weight of denervated and normal rats treated with clenbuterol of different doses

Gastrocnemius (D : P<0.001; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	1.497 ± 0.080^{c}	2.340 ± 0.097^{a}
0.2mg	1.725 ± 0.048^{bc}	2.352 ± 0.121^{a}
0.6mg	1.871 ± 0.046^{b}	2.238 ± 0.187^{a}
1.0mg	1.757 ± 0.015^{bc}	2.300 ± 0.076^{a}
2.0mg	1.739 ± 0.209^{bc}	2.232 ± 0.097^{a}

TA (D : P<0.001; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.540 ± 0.030^{d}	0.801 ± 0.057^{a}
0.2mg	0.594 ± 0.007^{cd}	0.810 ± 0.021^{a}
0.6mg	0.665 ± 0.032^{bc}	0.755 ± 0.032^{a}
1.0mg	0.634 ± 0.009^{cd}	0.791 ± 0.018^{a}
2.0mg	0.582 ± 0.064^{cd}	0.777 ± 0.043^{a}

EDL (D : P<0.001; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.172 ± 0.009^{e}	0.229 ± 0.013^{ab}
0.2mg	0.177 ± 0.007^{e}	0.215 ± 0.010^{abcd}
0.6mg	0.199 ± 0.021^{bcde}	0.223 ± 0.011^{abc}
1.0mg	0.194 ± 0.006^{cde}	0.232 ± 0.014^{a}
2.0mg	0.186 ± 0.018^{de}	0.211 ± 0.005^{abcd}

... to be continued

Table 4.8 (continued)

Soleus (D : P<0.001; C : N.S.; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.124 ± 0.006^{d}	0.188 ± 0.011^{a}
0.2mg	0.134 ± 0.009^{cd}	0.180 ± 0.005^{a}
0.6mg	0.147 ± 0.014^{cd}	0.171 ± 0.008^{ab}
1.0mg	0.143 ± 0.006^{cd}	0.188 ± 0.005^{a}
2.0mg	0.150 ± 0.008^{bc}	0.191 ± 0.006^{a}

Values are expressed in unit of gram.
Doses of clenbuterol used are in unit of mg/kg body-weight per day.
TA : Tibialis anterior; EDL : Extensor digitorum longus
Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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tigated, the muscle weights are shown in Table 10 and Table 11. Table 10 shows the muscle weight in rats with acute treatment while Table 11 shows that in rats with long term treatment. For the acute treated groups, denervation caused significant decrease in both percentage weight and absolute weight of gastrocnemius, TA and soleus but not that of EDL. Clenbuterol treatment did not cause any significant alteration in the muscle weights, both in control and

Table 4.9 Percentage muscle weight of denervated and normal rats treated with clenbuterol in different doses

Gastrocnemius (D : P<0.001; C : P<0.05; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.394 ± 0.018^{c}	0.568 ± 0.015^{a}
0.2mg	0.463 ± 0.015^{b}	0.610 ± 0.011^{a}
0.6mg	0.443 ± 0.027^{bc}	0.604 ± 0.018^{a}
1.0mg	0.461 ± 0.014^{b}	0.581 ± 0.016^{a}
2.0mg	0.434 ± 0.035^{bc}	0.566 ± 0.010^{a}

TA (D : P<0.001; C : P=0.054; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.142 ± 0.007^{c}	0.194 ± 0.006^{a}
0.2mg	0.160 ± 0.008^{bc}	0.211 ± 0.003^{a}
0.6mg	0.157 ± 0.008^{bc}	0.206 ± 0.008^{a}
1.0mg	0.166 ± 0.006^{b}	0.200 ± 0.008^{a}
2.0mg	0.145 ± 0.010^{c}	0.197 ± 0.006^{a}

EDL (D : P<0.001; C : P<0.05; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.045 ± 0.0006^{e}	0.055 ± 0.002^{bc}
0.2mg	0.048 ± 0.001^{de}	0.056 ± 0.001^{bc}
0.6mg	0.047 ± 0.002^{de}	0.061 ± 0.001^{a}
1.0mg	0.051 ± 0.002^{cd}	0.059 ± 0.003^{ab}
2.0mg	0.047 ± 0.003^{de}	0.054 ± 0.0006^{c}

... to be continued

Table 4.9 (continued)

Soleus (D : P<0.001; C : P=0.070; D*C : N.S.)

Dose	Denervated Rats	Control Rats
0.0mg	0.033 ± 0.002^{c}	0.046 ± 0.001^{a}
0.2mg	0.036 ± 0.001^{bc}	0.047 ± 0.0004^{a}
0.6mg	0.035 ± 0.002^{bc}	0.047 ± 0.001^{a}
1.0mg	0.038 ± 0.001^{b}	0.048 ± 0.001^{a}
2.0mg	0.038 ± 0.0003^{b}	0.049 ± 0.002^{a}

Values are expressed as percentage of body weight.
Doses of clenbuterol used are in unit of mg/kg body-weight per day.
TA : Tibialis anterior; EDL : Extensor digitorum longus
Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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denervated groups.

In the long term treatment experiment, denervation induced significant decrease in both the absolute and percentage weight of all the muscles investigated. In this experiment, significant effect of clenbuterol can also be observed in gastrocnemius and TA in which clenbuterol can partially restored the weight decreases inflicted by denervation while the drug alone has no effect. No

Table 4.10 Muscle weight of rats with acute treatment of denervation and clenbuterol

G	Absolute weight (g)		Percentage of body weight	
NS	2.293 ± 0.157[a]	D: P<0.001 C: N.S. D*C: N.S.	0.637 ± 0.033[a]	D: P<0.001 C: N.S. D*C: N.S.
NC	3.030 ± 0.060[a]		0.650 ± 0.013[a]	
DS	2.293 ± 0.108[b]		0.527 ± 0.025[b]	
DC	2.460 ± 0.044[b]		0.559 ± 0.010[b]	

T	Absolute weight (g)		Percentage of body weight	
NS	0.873 ± 0.018[a]	D: P<0.001 C: N.S. D*C: N.S.	0.186 ± 0.004[a]	D: P<0.005 C: N.S. D*C: N.S.
NC	0.891 ± 0.011[a]		0.191 ± 0.003[a]	
DS	0.717 ± 0.044[b]		0.165 ± 0.010[b]	
DC	0.771 ± 0.018[b]		0.175 ± 0.004[ab]	

E	Absolute weight (g)		Percentage of body weight	
NS	0.238 ± 0.013[a]	D: N.S. C: N.S. D*C: N.S.	0.051 ± 0.003[a]	D: N.S. C: N.S. D*C: N.S.
NC	0.249 ± 0.007[a]		0.053 ± 0.002[a]	
DS	0.220 ± 0.017[a]		0.051 ± 0.004[a]	
DC	0.237 ± 0.006[a]		0.054 ± 0.001[a]	

... to be continued

Table 4.10 (continued)

S	Absolute weight		Percentage of body weight	
NS	0.213 ± 0.008[a]	C: P<0.001 D: N.S. D*C: P=0.06	0.045 ± 0.002[a]	C: P<0.001 D: N.S. D*C: N.S.
NC	0.192 ± 0.004[b]		0.041 ± 0.001[ab]	
DS	0.160 ± 0.007[c]		0.037 ± 0.002[b]	
DC	0.166 ± 0.007[c]		0.038 ± 0.002[b]	

G ; gastrocnemius; T : tibialis anterior; E : extensor digitorum longus; S : soleus
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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influence of clenbuterol on EDL and soleus was observed.

The age effect of denervation and clenbuterol treatment was also examined. The result in adult rats and in young rats are shown in Table 4.12 and Table 4.13, respectively. In both batches of animals, denervation caused significant decline in both the absolute and percentage weights of all the muscles looked at. In the adult rats, clenbuterol increased the weight of gastrocnemius and soleus but the effect was not specific to denervated rats. Clenbuterol had

Table 4.11 Muscle weight of rats with long term treatment of denervation and clenbuterol

G	Absolute weight (g)		Percentage of body weight	
NS	2.586 ± 0.096^{b}	D: P<0.001 C: P<0.001 D*C: N.S.	0.528 ± 0.020^{a}	D: P<0.001 C: P<0.001 D*C: P=0.06
NC	2.739 ± 0.068^{a}		0.553 ± 0.014^{a}	
DS	1.259 ± 0.026^{d}		0.274 ± 0.006^{c}	
DC	1.600 ± 0.024^{c}		0.340 ± 0.005^{b}	

T	Absolute weight (g)		Percentage of body weight	
NS	0.943 ± 0.026^{a}	D: P<0.001 C: P<0.025 D*C: P<0.001	0.193 ± 0.005^{a}	D: P<0.001 C: P=0.059 D*C: P<0.001
NC	0.925 ± 0.027^{a}		0.187 ± 0.006^{a}	
DS	0.419 ± 0.009^{c}		0.091 ± 0.002^{c}	
DC	0.521 ± 0.010^{b}		0.111 ± 0.002^{b}	

E	Absolute weight (g)		Percentage of body weight	
NS	0.256 ± 0.009^{a}	D: P<0.001 C: N.S. D*C: N.S.	0.052 ± 0.002^{a}	D: P<0.001 C: N.S. D*C: N.S.
NC	0.255 ± 0.007^{a}		0.051 ± 0.001^{a}	
DS	0.139 ± 0.003^{b}		0.030 ± 0.001^{b}	
DC	0.155 ± 0.006^{b}		0.033 ± 0.001^{b}	

... to be continued

Table 4.11 (continued)

S	Absolute weight (g)		Percentage of body weight	
NS	0.213 ± 0.006[a]	D: P<0.001 C: N.S. D*C: N.S.	0.044 ± 0.001[a]	D: P<0.001 C: N.S. D*C: N.S.
NC	0.213 ± 0.009[a]		0.043 ± 0.002[a]	
DS	0.109 ± 0.002[c]		0.024 ± 0.000[c]	
DC	0.124 ± 0.003[b]		0.026 ± 0.001[b]	

G ; gastrocnemius; T : tibialis anterior; E : extensor digitorum longus; S : soleus
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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no effect on EDL and TA, either when used alone or on denervated animals. In the young rats, clenbuterol had no effect when used alone but the drug is capable of restoring partially the weight losses of denervated gastrocnemius, TA and soleus, but not of EDL.

On the whole, it was found that the effect of denervation on muscle weight, both in terms of the absolute and the percentage, was very marked. However, EDL responded to denervation slowly and thus its weight did not change

Table 4.12 Muscle weight of adult rats with denervation and clenbuterol treatment

G	Absolute weight (g)		Percentage of body weight	
NS	2.280 ± 0.069[a]	D: P<0.001 C: P<0.05 D*C: N.S.	0.555 ± 0.010[a]	D: P<0.001 C: P<0.05 D*C: N.S.
NC	2.375 ± 0.053[a]		0.569 ± 0.008[a]	
DS	1.594 ± 0.033[b]		0.403 ± 0.006[c]	
DC	1.702 ± 0.033[b]		0.431 ± 0.012[b]	

T	Absolute weight (g)		Percentage of body weight	
NS	0.786 ± 0.020[a]	D: P<0.001 C: N.S. D*C: N.S.	0.191 ± 0.003[a]	D: P<0.001 C: N.S. D*C: N.S.
NC	0.822 ± 0.020[a]		0.197 ± 0.003[a]	
DS	0.583 ± 0.011[b]		0.147 ± 0.002[b]	
DC	0.598 ± 0.017[b]		0.151 ± 0.004[b]	

E	Absolute weight (g)		Percentage of body weight	
NS	0.212 ± 0.005[a]	D: P<0.001 C: N.S. D*C: N.S.	0.052 ± 0.001[a]	D: P<0.001 C: N.S. D*C: N.S.
NC	0.217 ± 0.005[a]		0.052 ± 0.001[a]	
DS	0.178 ± 0.005[b]		0.045 ± 0.001[b]	
DC	0.169 ± 0.004[b]		0.043 ± 0.001[b]	

... to be continued

Table 4.12 (continued)

S	Absolute weight (g)		Percentage of body weight	
NS	0.176 ± 0.004[a]	D: P<0.001 C: P<0.025 D*C: N.S.	0.043 ± 0.001[a]	D: P<0.001 C: P<0.025 D*C: N.S.
NC	0.187 ± 0.005[a]		0.045 ± 0.001[a]	
DS	0.119 ± 0.004[b]		0.030 ± 0.001[b]	
DC	0.128 ± 0.004[b]		0.032 ± 0.001[b]	

G ; gastrocnemius; T : tibialis anterior; E : extensor digitorum longus; S : soleus
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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significantly 4 days after denervation. From the long term effect of clenbuterol on gastrocnemius and TA, it may be concluded that these muscles may respond to a low dose (0.2 mg/kg body-weight per day) of clenbuterol only when the period of administration is long enough. With comparison to the muscle weight of the animals treated with a higher dose (0.6 mg/kg body-weight per day) of clenbuterol for one week (see Table 4.12), it was suggested that soleus responds

Table 4.13 Muscle weight of young rats with denervation and clenbuterol treatment

G	Absolute weight (g)		Percentage of body weight	
NS	0.744 ± 0.015[a]	D: P<0.001 C: P<0.001 D*C: P<0.05	0.480 ± 0.010[a]	D: P<0.001 C: P<0.005 D*C: P<0.005
NC	0.780 ± 0.022[a]		0.481 ± 0.009[a]	
DS	0.444 ± 0.012[c]		0.286 ± 0.004[c]	
DC	0.545 ± 0.013[b]		0.329 ± 0.006[b]	

T	Absolute weight (g)		Percentage of body weight	
NS	0.307 ± 0.015[a]	D: P<0.001 C: P<0.05 D*C: P<0.05	0.198 ± 0.008[a]	D: P<0.001 C: N.S. D*C: P<0.005
NC	0.308 ± 0.008[a]		0.189 ± 0.003[a]	
DS	0.174 ± 0.005[c]		0.112 ± 0.002[c]	
DC	0.216 ± 0.006[b]		0.130 ± 0.003[b]	

E	Absolute weight (g)		Percentage of body weight	
NS	0.082 ± 0.003[a]	D: P<0.001 C: N.S. D*C: N.S.	0.053 ± 0.001[a]	D: P<0.001 C: N.S. D*C: N.S.
NC	0.084 ± 0.004[a]		0.052 ± 0.002[a]	
DS	0.062 ± 0.002[b]		0.040 ± 0.001[b]	
DC	0.066 ± 0.002[b]		0.040 ± 0.001[b]	

... to be continued

Table 4.13 (continued)

S	Absolute weight (g)		Percentagr of body weight	
NS	0.070 ± 0.002[a]	D: P<0.001 C: P<0.001 D*C: P<0.001	0.045 ± 0.002[a]	D: P<0.001 C: N.S. D*C: P<0.001
NC	0.070 ± 0.002[a]		0.043 ± 0.002[a]	
DS	0.038 ± 0.001[c]		0.024 ± 0.001[c]	
DC	0.052 ± 0.002[b]		0.031 ± 0.001[b]	

G ; gastrocnemius; T : tibialis anterior; E : extensor digitorum longus; S : soleus
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

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better to a higher dose of clenbuterol than a longer treatment period. However, TA responds better to a long administration period, instead.

In young rats, with the exception of EDL, muscles are very sensitive to clenbuterol treatment. Also, the interaction effect of denervation and clenbuterol treatment which was hardly observed in adult rats was also significant in the muscles of young rats.

Body weight and muscle weight in the experimental animals illustrated the localized effect of clenbuterol on rats. The body weights of both adult and young rats did not respond to clenbuterol administration but the muscle weights increased significantly. Also, clenbuterol effect may be age-dependent. Clenbuterol affected the organ weight of young rats but not that of adult rats.

4.3.2 Biochemical Changes

4.3.2.1 Energy Metabolizing Enzymes

The alteration of metabolic activities in denervated muscles has been studied rather extensively (Turner and Manchester, 1972; Nemeth et al., 1980; Max et al., 1981; Shackelford and Lebherz, 1981; Evans, 1983; Moruzzi and Bergamini, 1983; Asotra and Asotra, 1984; Simard et al., 1985; Shoji, 1986). But the effect of clenbuterol on the metabolism of denervated muscles has not been reported.

Two energy metabolizing enzymes were studied in our experiment. They were malate dehydrogenase (MDH) and lactate dehydrogenase (LDH). MDH is a component enzyme of the citric acid cycle and is regarded as the representative of enzymes involved in oxidative metabolism. LDH is the enzyme involved in the final step of anaerobic glycolysis and its level may reflect the activity of this process.

In rats subjected to acute treatment, denervation caused significant effect on both MDH specific and total activity of all the muscles studied (Figure 4.1). In

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.005	P<0.05	N.S.
	Total	P<0.001	N.S.	N.S.
EDL	Specific	P<0.005	P<0.05	N.S.
	Total	P<0.005	P=0.053	N.S.
Soleus	Specific	P<0.005	P<0.01	N.S.
	Total	P<0.005	P<0.005	P<0.01

Figure 4.1 MDH activities in muscles of rats with acute denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day and the treatment was lasted for 4 days. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Soleus
0
2
4
6
8
10
50
100
150
200
1000
2000
3000

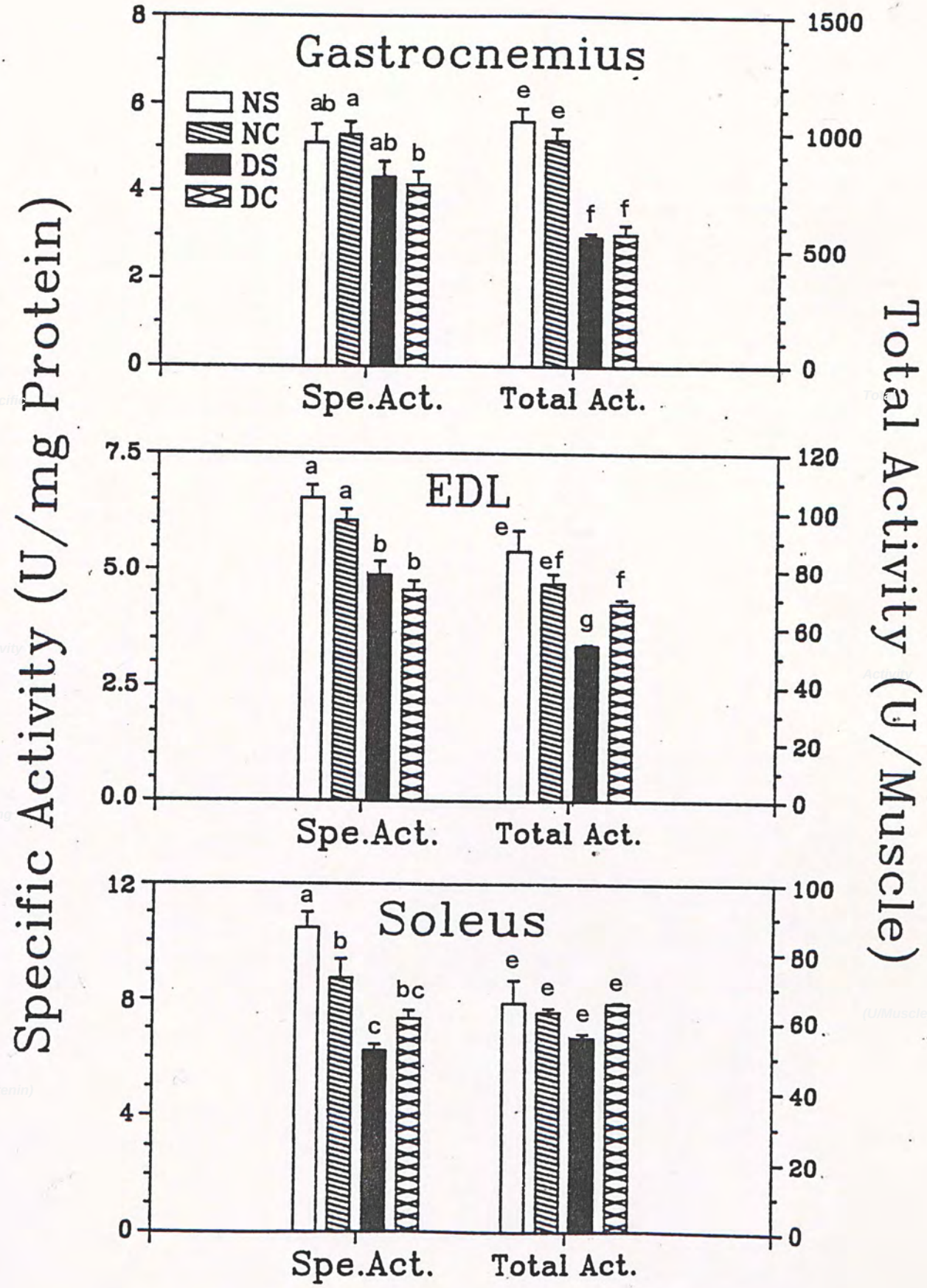
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	N.S.	N.S.
	Total	P<0.001	P<0.001	P<0.025
EDL	Specific	P<0.005	N.S.	N.S.
	Total	P<0.001	N.S.	N.S.
Soleus	Specific	P<0.01	P<0.05	N.S.
	Total	P<0.001	N.S.	N.S.

Figure 4.2 MDH activities in muscles of rats with chronic denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Soleus
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

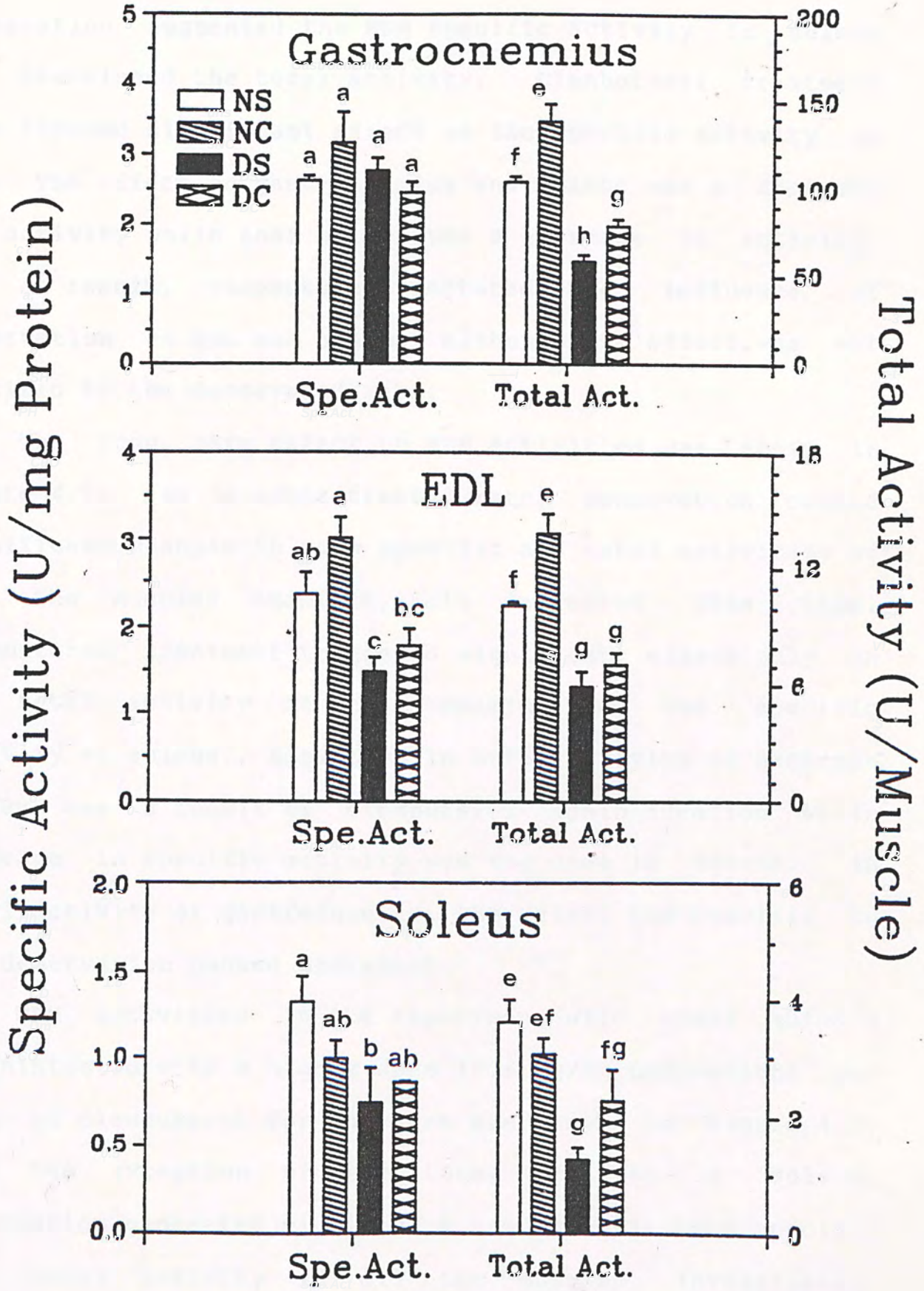
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.05	N.S.	N.S.
	Total	P<0.001	N.S.	N.S.
EDL	Specific	P<0.001	N.S.	N.S.
	Total	P<0.005	N.S.	P<0.025
Soleus	Specific	P<0.001	N.S.	P<0.025
	Total	N.S.	N.S.	N.S.

Figure 4.3 MDH activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
ab a ab b
e e f f
8
6
4
2
0
1500
1000
500
0
Spe.Act.
Total Act.
EDL
a a b b
e ef g f
7.5
5.0
2.5
0.0
120
100
80
60
40
20
0
Spe.Act.
Total Act.
Soleus
a b c bc
e e e e
12
8
4
0
100
80
60
40
20
0
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	N.S.
	Total	P<0.001	P<0.005	N.S.
EDL	Specific	P<0.005	P=0.052	N.S.
	Total	P<0.001	P<0.025	N.S.
Soleus	Specific	P<0.05	N.S.	N.S.
	Total	P<0.01	N.S.	N.S.

Figure 4.4 MDH activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Soleus
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

gastrocnemius and EDL, both specific and total activities declined significantly after denervation. However, denervation augmented the MDH specific activity in soleus but diminished the total activity. Clenbuterol treatment also induced significant effect on the specific activity of MDH. The effect on gastrocnemius and soleus was a decrease in activity while that on EDL was a increase in activity. As a result, clenbuterol restored the influence of denervation on EDL and soleus, although the effect was not specific to the denervated rats.

The long term effect on MDH activities was shown in Figure 4.2. As in acute treated rats, denervation caused significant changes in both specific and total activities of all the muscles examined, all decreased this time. Clenbuterol treatment triggered significant effect only in the total activity of gastrocnemius and the specific activity of soleus. Elevation in total activity of gastrocnemius was a result of clenbuterol administration while decrease in specific activity was the case in soleus. In total activity of gastrocnemius, the effect was specific to the denervation caused decrement.

MDH activities in the experiment with adult animals administrated with a higher dose (0.6 mg/kg body-weight per day) of clenbuterol for one week are shown in Figure 4.3. With the exception of the total activity in soleus, denervation generated significant reduction in both specific and total activity in all the muscles investigated. Clenbuterol treatment alone exerted no significant effect on

all the data shown. Clenbuterol appeared to restore the denervation decreased enzyme activity partially in the total activity of EDL and the specific activity of soleus.

Figure 4.4 shows the MDH activity in young rats. Denervation caused a decrease in all the activities examined with the exception of specific activity in gastrocnemius. Clenbuterol administration had no effect on the activity in soleus. Clenbuterol increased both the specific and the total activities in EDL and the total activity in gastrocnemius. No effect of clenbuterol detected was specific to denervation.

On the whole, denervation caused significant change in the MDH activities with the exception in young rat gastrocnemius. In gastrocnemius of adult rats, decreased MDH activities appeared not later than the fourth day after the operation. In EDL, a reduced MDH activity was found in all situations investigated. However, specific MDH activity in soleus increased on the fourth day after the operation and decreased at the following time examined. This may reflect the fact that soleus, a slow twitch muscle which obtained its energy mainly from oxidative metabolism, responses to denervation differently from the fast twitch muscles.

Lower specific MDH activity was found in adult gastrocnemius after the acute treatment with clenbuterol. This change disappeared when the treatment was maintained. After long term administration, however, clenbuterol increased the total activity of MDH and thus reversed the effect exerted

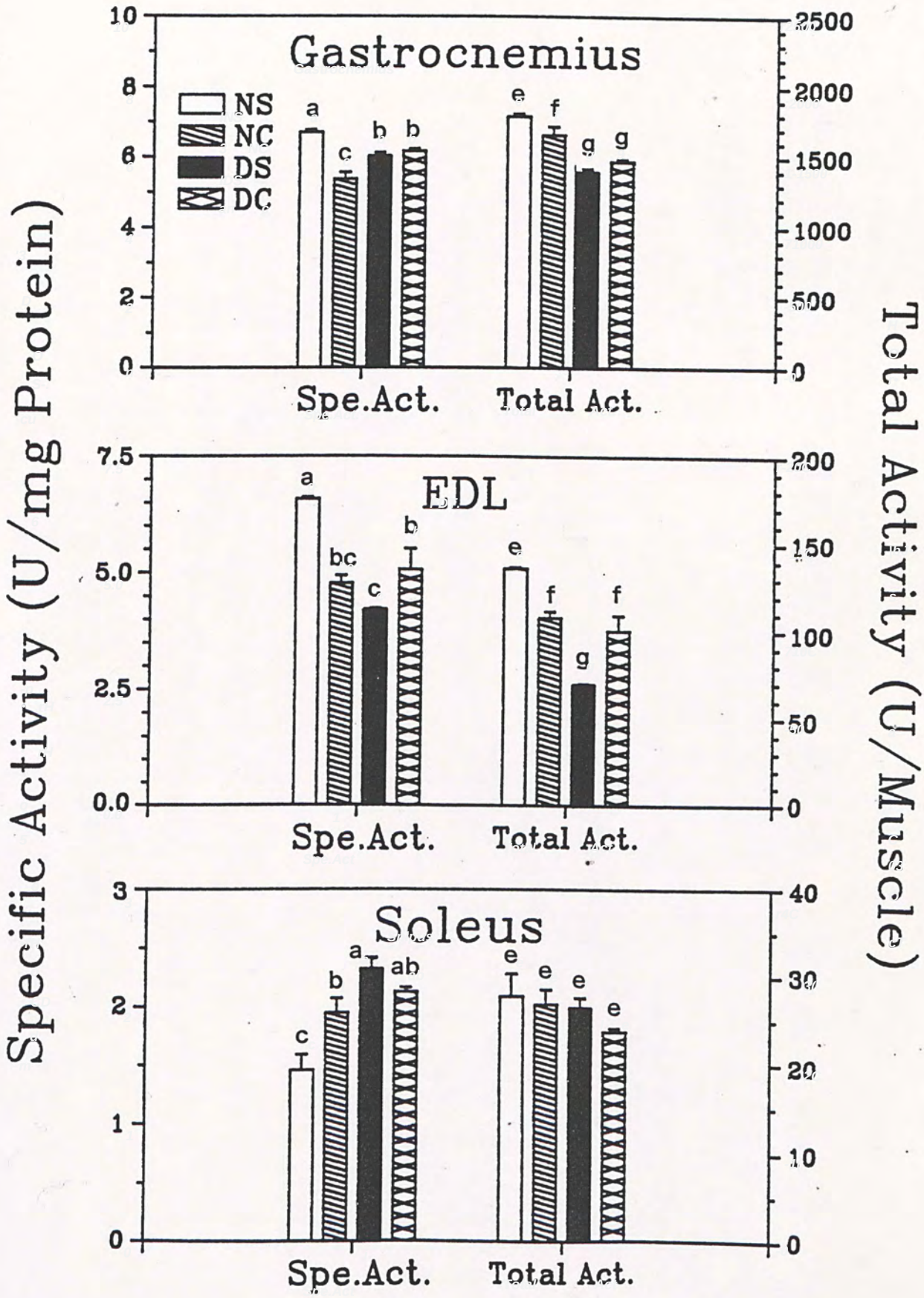
by denervation on this muscle. In the young rats, clenbuterol administration also induced an increased level of total MDH activity in gastrocnemius. Effect of clenbuterol administration on EDL was mainly an increase in the MDH activity. With the exception of the long term treated rats, elevated MDH activity appeared in EDL after clenbuterol administration. Administration of a large dose of clenbuterol for one week increased and restored the denervation caused decrease in total MDH activity in EDL. In soleus, MDH activity decreased when a low dose of clenbuterol was used but increased and was restored partially to its normal value when a high dose was used.

Another energy metabolizing enzyme assayed was LDH. LDH activities in acutely treated rats are shown in Figure 4.5. Denervation decreased the enzyme activity in control gastrocnemius and EDL significantly. In gastrocnemius, clenbuterol lessened the enzyme activity only in the control muscles. In EDL, clenbuterol increased LDH activity in the denervated muscles but decreased it in the control muscles. As a result, clenbuterol administration restored partially the decrement caused by denervation. Increased specific LDH activity was found in denervated soleus. Only the specific activity in control soleus was elevated significantly.

For the long term treated animals, LDH activities are shown in Figure 4.6. Denervation caused significant decrease in both specific and total activities in gastroc-

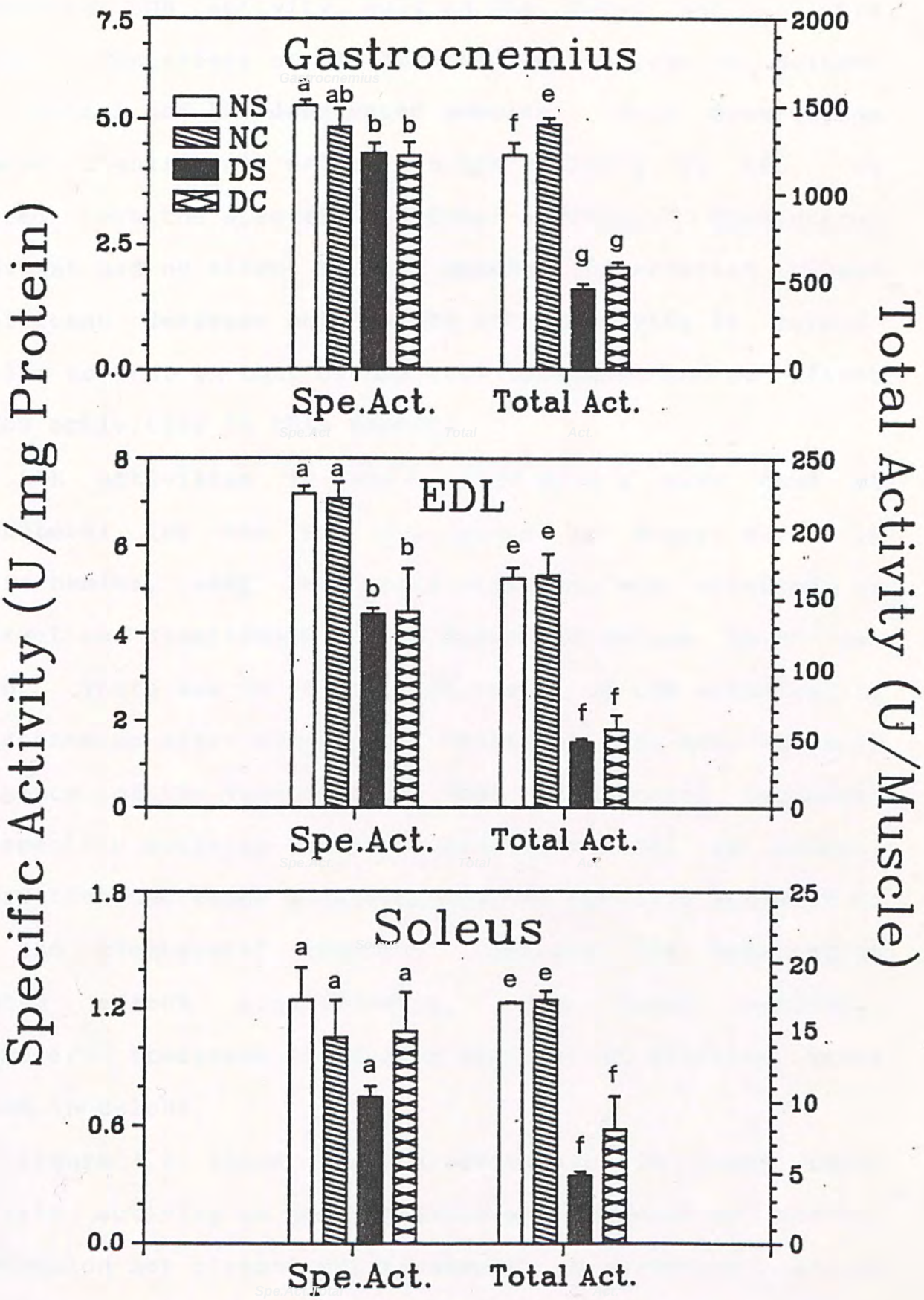
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	P<0.005	P<0.001
	Total	P<0.001	N.S.	P<0.025
EDL	Specific	P<0.005	P<0.05	P<0.005
	Total	P<0.001	N.S.	P<0.001
Soleus	Specific	P<0.005	N.S.	P<0.01
	Total	N.S.	N.S.	N.S.

Figure 4.5 LDH activities in muscles of rats with acute denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Spe.Act.
Total Act.
EDL
Soleus

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.05	N.S.	N.S.
	Total	P<0.001	P<0.005	N.S.
EDL	Specific	P<0.01	N.S.	N.S.
	Total	P<0.001	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	P<0.001	N.S.	N.S.

Figure 4.6 LDH activities in muscles of rats with chronic denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Spe.Act.
Total Act.
EDL
Soleus
7.5
5.0
2.5
0.0
2000
1500
1000
500
0
8
6
4
2
250
200
150
100
50
1.8
1.2
0.6
25
20
15
10
5

nemius. Clenbuterol treatment expressed its effect of increasing LDH activity only on the total one in this muscle. The effect of clenbuterol was specific to neither the control nor the denervated muscles. Only denervation induced significant effect on LDH activity in EDL. It lowered both the specific and total activity. Clenbuterol treatment had no effect on this muscle. Denervation caused significant decrease only in LDH total activity in soleus. Similar to that in EDL, clenbuterol treatment had no effect on LDH activities in this muscle.

LDH activities in rats treated with a high dose of clenbuterol for one week are shown in Figure 4.7. In gastrocnemius, only the total activity was affected by denervation significantly. A decreased enzyme level was found. There was no significant change of LDH activity in gastrocnemius after clenbuterol treatment. In EDL, the sole influence of the treatment was that clenbuterol decreased the specific activity in the denervated muscle. In soleus, denervation decreased significantly the specific activity of LDH and clenbuterol treatment reversed the denervation induced effect significantly. For total activity, clenbuterol treatment elicited a significant elevated level of LDH in soleus.

Figure 4.8 shows the LDH activities in young rats. Specific activity in gastrocnemius was affected by neither denervation nor clenbuterol treatment. Denervation reduced but clenbuterol treatment elevated the total activity of LDH

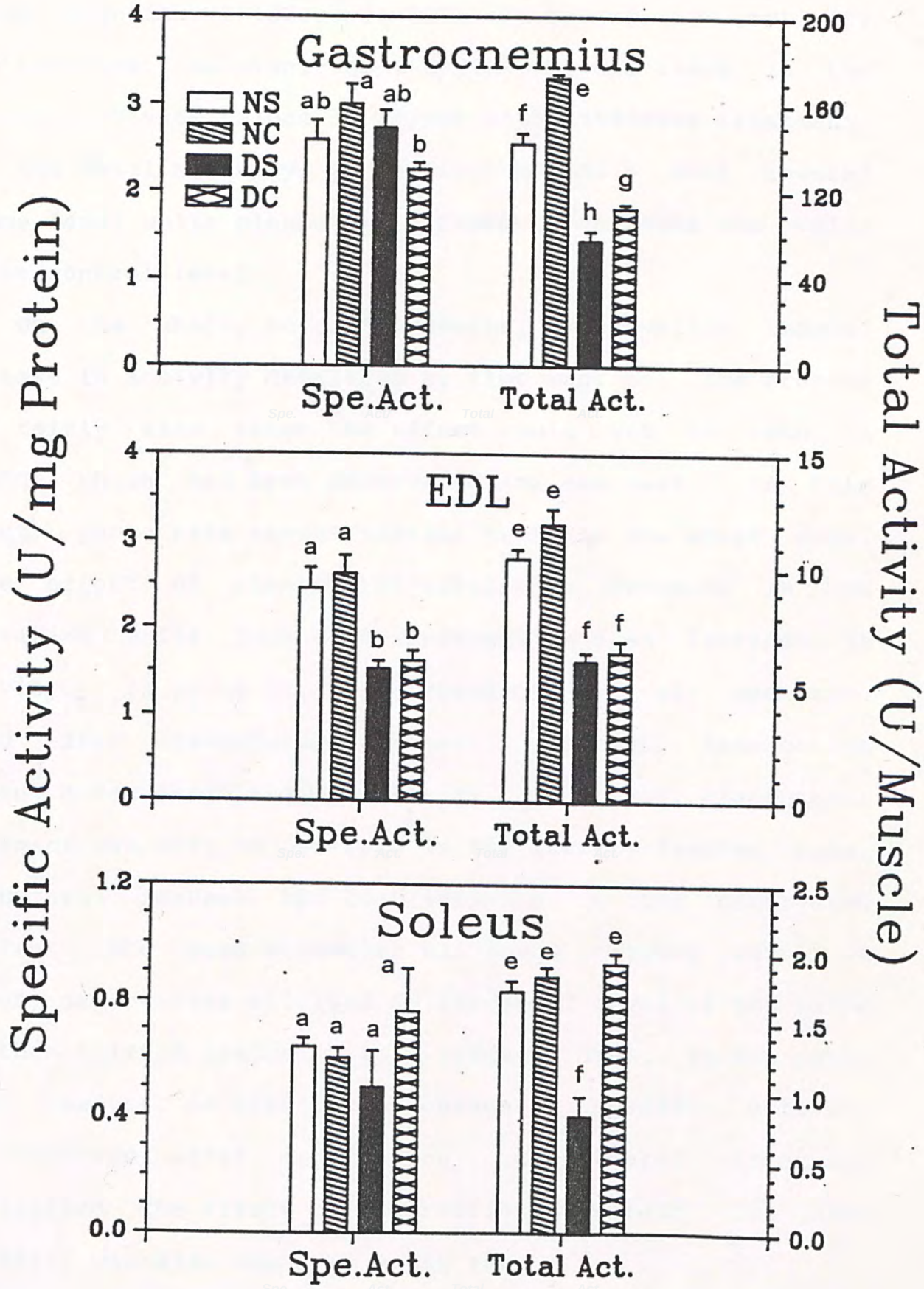
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	N.S.
	Total	P<0.005	N.S.	N.S.
EDL	Specific	N.S.	N.S.	P<0.05
	Total	N.S.	N.S.	N.S.
Soleus	Specific	P<0.01	N.S.	P<0.025
	Total	N.S.	P=0.056	N.S.

Figure 4.7 LDH activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Soleus

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	P=0.052
	Total	P<0.001	P<0.001	P=0.055
EDL	Specific	P<0.001	N.S.	N.S.
	Total	P<0.001	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	P<0.005	P<0.001	P<0.005

Figure 4.8 LDH activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
ab
a
ab
b
f
e
h
g
Spe.Act.
Total Act.
EDL
a
a
b
b
e
e
f
f
Spe.Act.
Total Act.
Soleus
a
a
a
a
e
e
f
e
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
0
1
2
3
4
0
40
120
160
200
0
5
10
15
0.0
0.4
0.8
1.2
0.0
0.5
1.0
1.5
2.0
2.5

in gastrocnemius. In the factors studied, only denervation caused significant effect in EDL. Decreased LDH activity was resulted. No significant difference was found in the specific activity values in soleus with different treatment. For the total activity, Denervation caused a much lowered enzyme level while clenbuterol treatment restored the value to the control level.

On the whole, in gastrocnemius, denervation induced decrease in activity developed as time went on. The process was fairly slow since the effect could not be seen in muscles which had been denervated for one week. In this aspect, young rats showed similar trend as the adult ones. Acute effect of clenbuterol elicited a decrease in LDH activities while long term treatment gave an increase in activity. In young rats, increased LDH activity was also found after clenbuterol treatment. In EDL, denervation caused a decreased enzyme activity. Effect of clenbuterol treatment can only be observed in the acutely treated rats. Clenbuterol resumed the lost activity in the denervated muscles. The same situation was found in young rat. In soleus, denervation elicited an increased level of LDH first and then shifted gradually to a reduced level. In the young rats, however, no significant change in specific activity was observed after denervation. Clenbuterol treatment ameliorated the effect of denervation on soleus. The same situation was also found in young rats.

For the energy metabolizing enzymes investigated, it

was found that the effect of denervation and clenbuterol treatment was fairly similar in the same muscles.

4.3.2.2 Proteinases

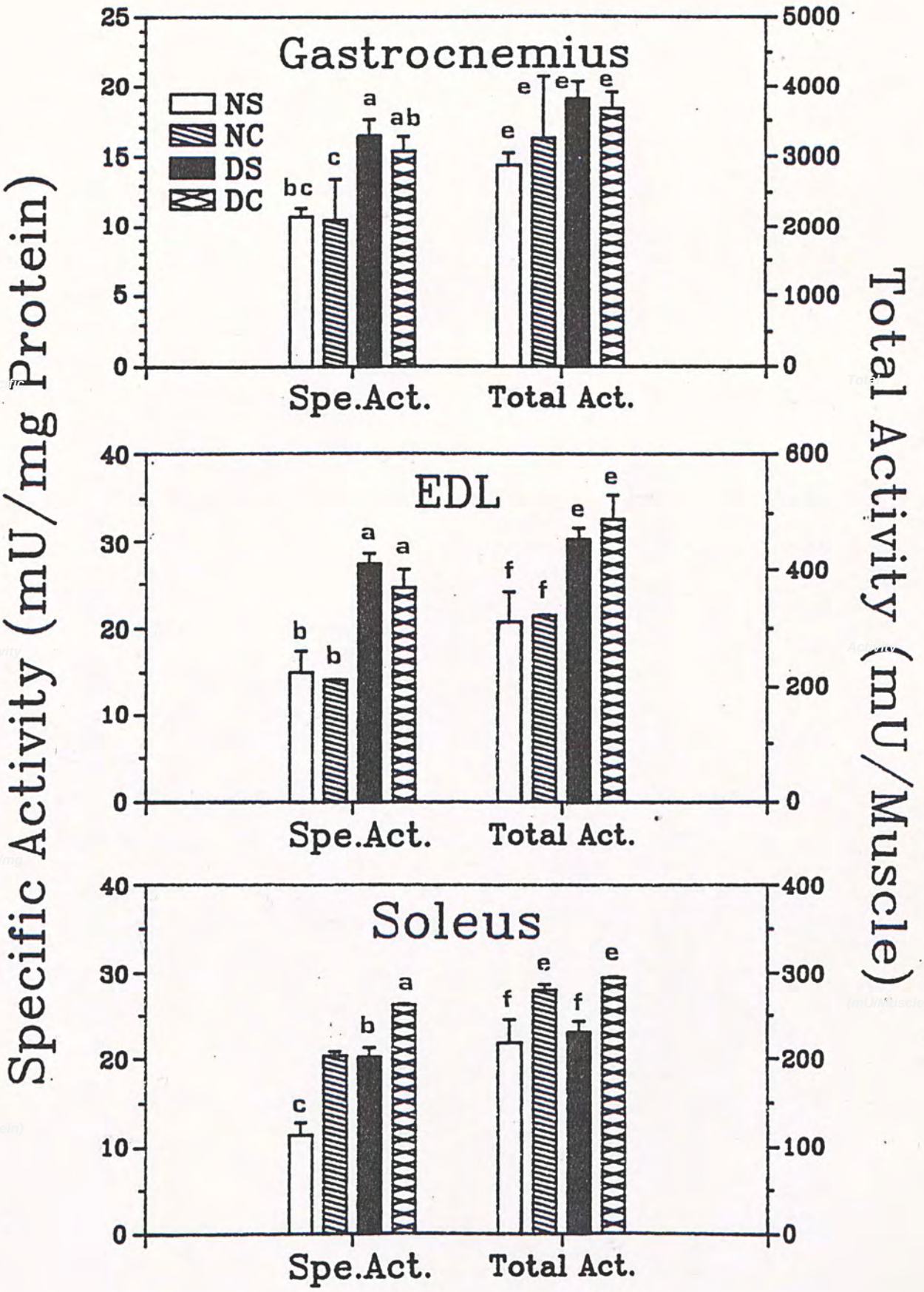
Denervation caused atrophy is related to a higher degree of protein degradation (Goldspink, 1976) which is usually a consequence of increased proteolytic activity. Also, it was reported that clenbuterol reversed the atrophy through both a higher protein synthesis rate and a lower protein degradation rate in denervated muscles (Maltin et al., 1987b). For this reason, the proteinase activities in the muscle homogenate of the treated rats were assayed to see if clenbuterol treatment exerted an effect on the proteinase activities.

First of all, activity of acid proteinase was determined. Increased specific acid proteinase activity was found in the muscle of acutely treated rats (Figure 4.9). For total activity, denervation caused significant effect only in EDL. Clenbuterol treatment increased acid proteinase activity only in soleus. The same effect of clenbuterol was found in both denervated and control muscles.

The long term effect of clenbuterol treatment on acid proteinase activity is shown in Figure 4.10. As in the acute treated rats, denervation elicited significant elevation of specific activity in all the muscles examined. Clenbuterol treatment had its effect on specific activity only in EDL. The influence of clenbuterol was specific to

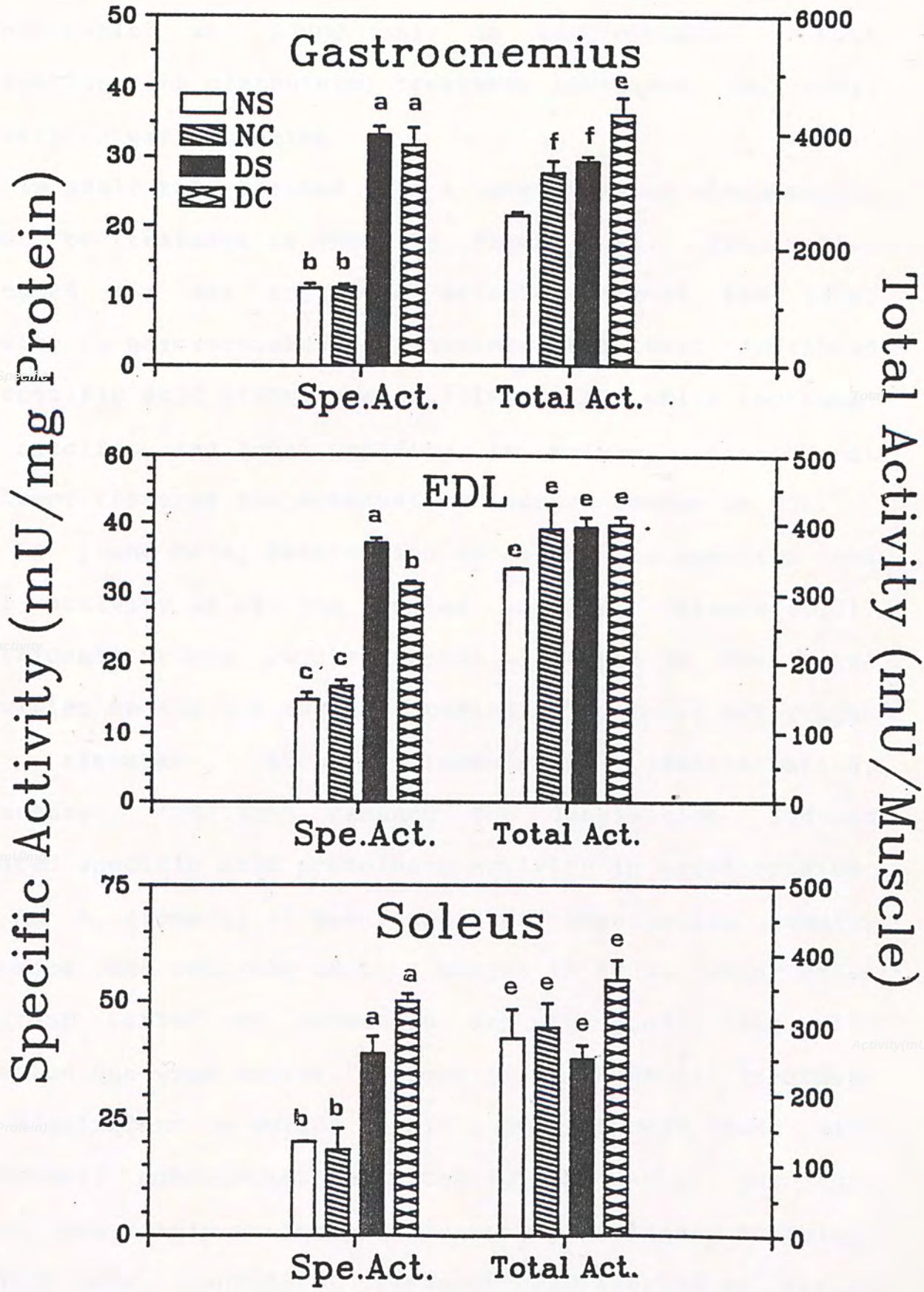
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.025	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
EDL	Specific	P<0.001	N.S.	N.S.
	Total	P<0.005	N.S.	N.S.
Soleus	Specific	P<0.001	P<0.001	N.S.
	Total	N.S.	P<0.005	N.S.

Figure 4.9 Acid proteinase activities in muscles of rats with acute denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)
Spe.Act.
Total Act.
EDL
Soleus

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	N.S.	N.S.
	Total	P<0.001	P<0.005	N.S.
EDL	Specific	P<0.001	N.S.	P<0.01
	Total	N.S.	N.S.	N.S.
Soleus	Specific	P<0.001	N.S.	N.S.
	Total	N.S.	N.S.	N.S.

Figure 4.10 Acid proteinase activities in muscles of rats with chronic denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)
Spe.Act.
Total Act.
EDL
Soleus

the denervation induced increased enzyme level. For the total activity, significant effect of the clenbuterol administration was found only in gastrocnemius. Both denervation and clenbuterol treatment increased the total activity in gastrocnemius.

In adult rats treated with a large dose of clenbuterol, effect of treatment is shown in Figure 4.11. Denervation increased all the activities detected except the total activity in gastrocnemius. Clenbuterol treatment decreased the specific acid proteinase activity in EDL while increased the specific and total activity in soleus. Clenbuterol treatment restored the denervation induced change in EDL.

In young rats, denervation increased the specific and total activity in all the muscles examined (Figure 4.12). Significant effect of clenbuterol was found in the total activities in all the muscles studied. The total activities were elevated after clenbuterol administration. Clenbuterol treatment resumed the denervation induced elevated specific acid proteinase activity in gastrocnemius.

As a summary, it was found that denervation usually increased the activity of this enzyme in adult rats after different period of operation and in young rats with operation one week before. Effect of clenbuterol treatment was significant in soleus of all animals except those with clenbuterol administration lasted for two weeks. But this effect was mainly an increase in acid proteinase activity. In adult rats, clenbuterol treatment only exerted an effect specific to the denervation caused change in EDL. This

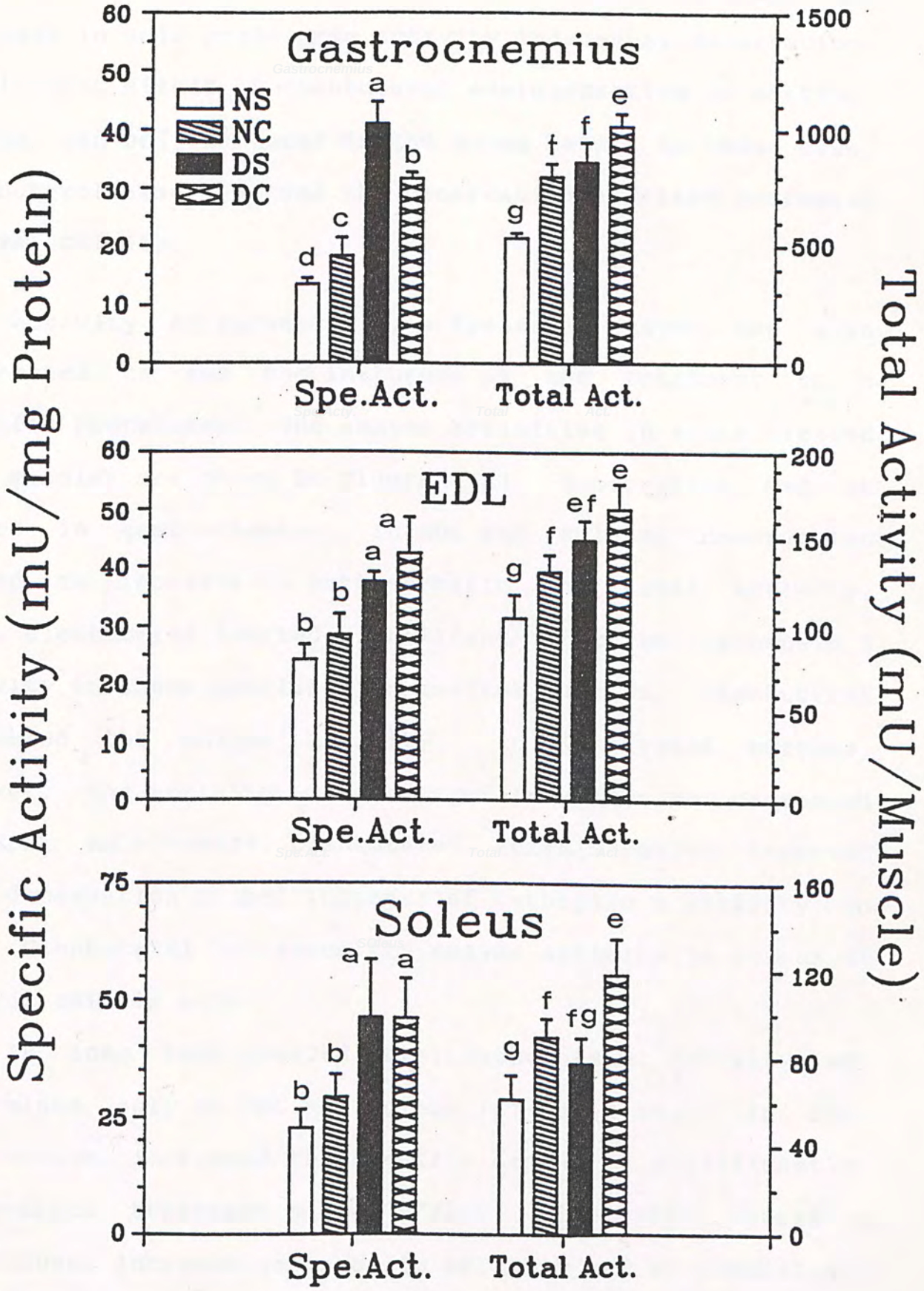
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.025	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
EDL	Specific	P<0.001	P<0.025	P<0.01
	Total	P<0.001	N.S.	N.S.
Soleus	Specific	P<0.001	P<0.025	N.S.
	Total	P<0.001	P<0.01	N.S.

Figure 4.11 Acid proteinase activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)
0
20
40
60
0
1000
2000
3000
4000
5000
0
100
200
300
400
500

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	N.S.	P<0.025
	Total	P<0.001	P<0.001	N.S.
EDL	Specific	P<0.001	N.S.	N.S.
	Total	P<0.001	P<0.025	N.S.
Soleus	Specific	P<0.005	N.S.	N.S.
	Total	P<0.05	P<0.005	N.S.

Figure 4.12 Acid proteinase activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Soleus
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)

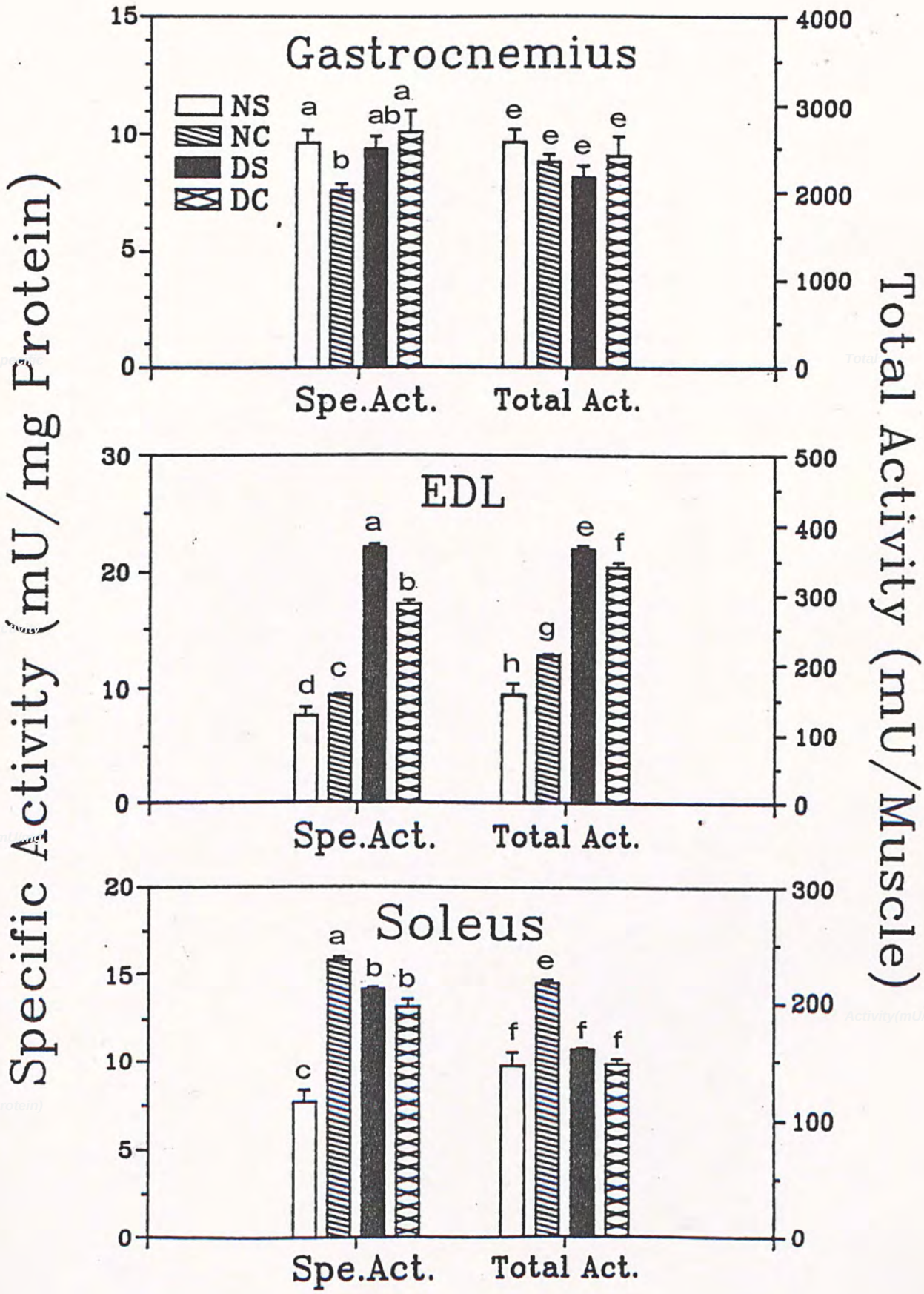
effect can only be observed when the treatment was lasted for over one week. Clenbuterol treatment reversed the increase in acid proteinase activity induced by denervation. Significant effect of clenbuterol administration in gastrocnemius can only be found in the young rats. In this case, clenbuterol also reversed the denervation elicited increased enzyme activity.

Activity of cathepsin B, a lysosomal enzyme, was also determined to see the influence of the treatment on a specific proteinase. The enzyme activities in acute treated rat muscles are shown in Figure 4.13. Denervation had no effect in gastrocnemius. In EDL and soleus, denervation caused an increase in both specific and total activity. Also, clenbuterol exerted significant effect on cathepsin B activity in these muscles. In control muscles, clenbuterol increased the enzyme activity. In denervated muscles, however, the activity was unchanged in soleus or decreased in EDL. As a result, clenbuterol administration inverted the denervation caused increase of cathepsin B activity in EDL. Clenbuterol increased the enzyme activity in soleus of control animals only.

In long term treated rats, cathepsin B activity was determined only in EDL and soleus (Figure 4.14). In EDL, denervation increased the specific activity significantly. Clenbuterol treatment had no effect. Denervation caused a significant increase in specific activity and a significant decrease in the total activity in soleus. The effect of

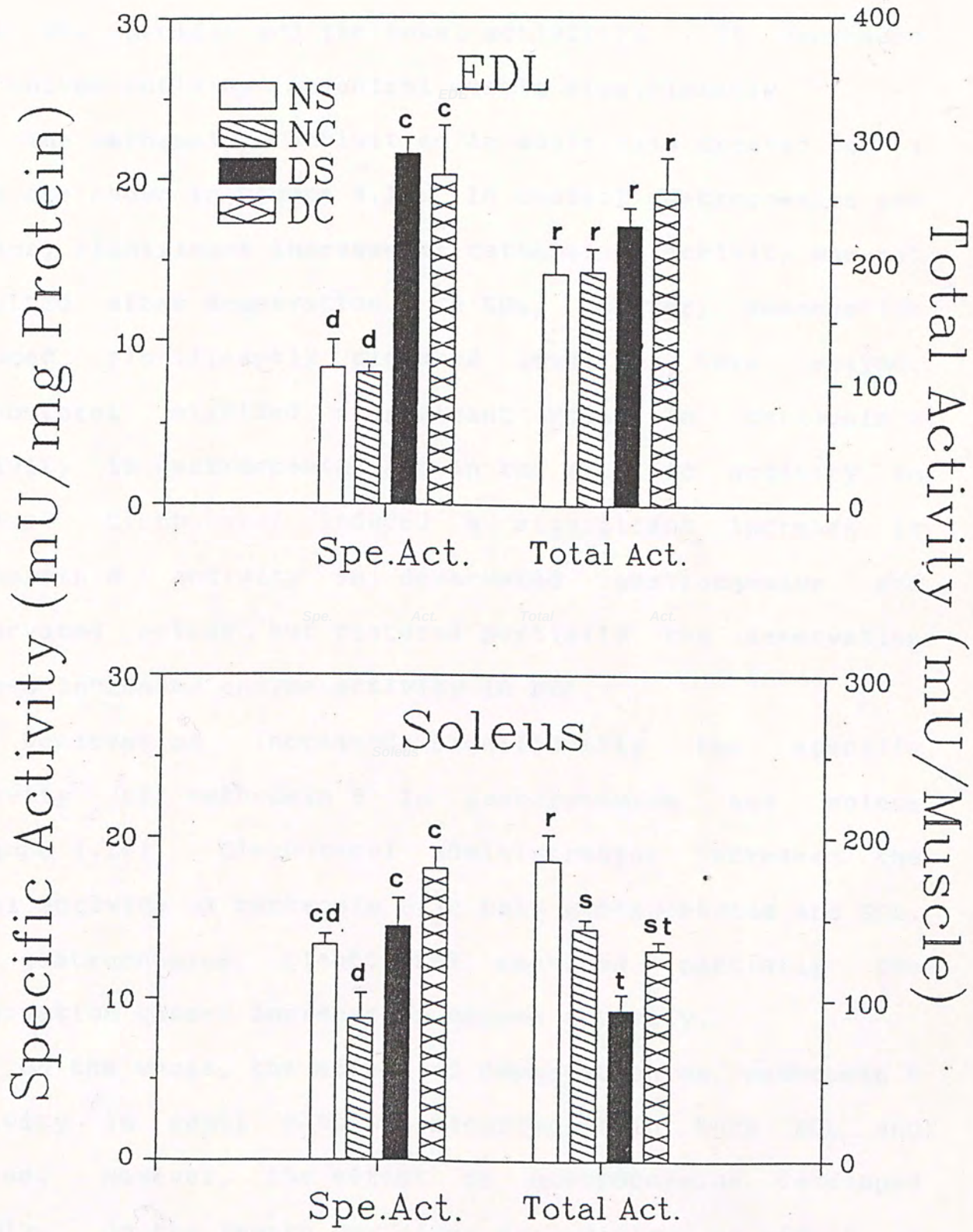
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	P<0.05
	Total	N.S.	N.S.	N.S.
EDL	Specific	P<0.001	P<0.01	P<0.001
	Total	P<0.001	N.S.	P<0.005
Soleus	Specific	P<0.005	P<0.001	P<0.001
	Total	P<0.005	P<0.005	P<0.001

Figure 4.13 Cathepsin B activities in muscles of rats with acute denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)

Muscles	Activity	D	C	D*C
EDL	Specific	P<0.005	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	P<0.025	N.S.	P<0.05
	Total	P<0.01	N.S.	P<0.025

Figure 4.14 Cathepsin B activities in muscles of rats with chronic denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


EDL
NS
NC
DS
DC
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.

clenbuterol treatment was specific to the control muscles in both the specific and the total activities. It decreased the enzyme activity in control muscle significantly.

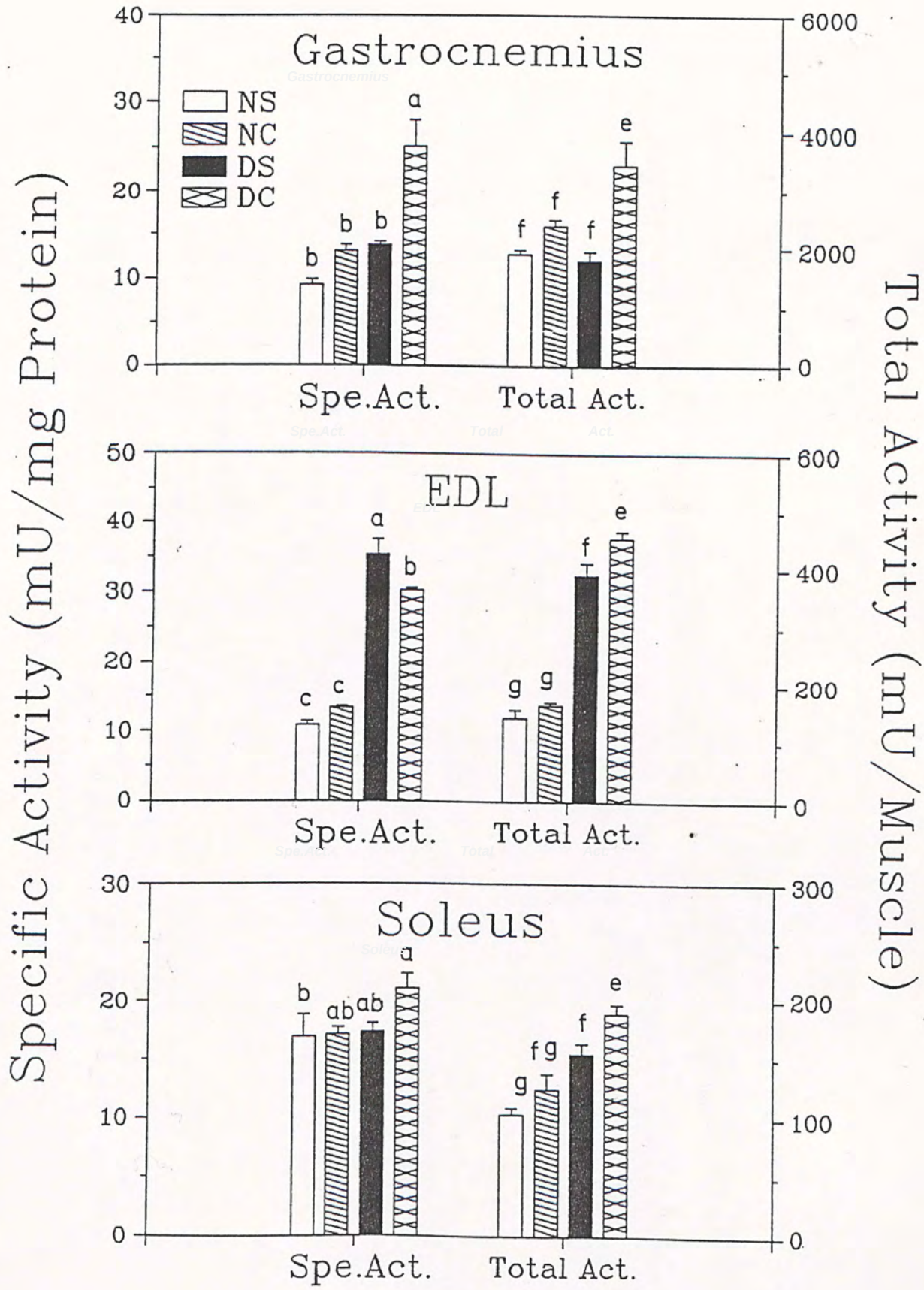
The cathepsin B activities in adult rats treated for a week are shown in Figure 4.15. In control gastrocnemius and soleus, significant increase of cathepsin B activity was not resulted after denervation. In EDL, however, denervation induced significantly elevated level of this enzyme. Clenbuterol elicited significant rise in cathepsin B activity in gastrocnemius and in the specific activity in soleus. Clenbuterol induced a significant increase in cathepsin B activity in denervated gastrocnemius and denervated soleus but restored partially the denervation caused increased enzyme activity in EDL.

Denervation increased significantly the specific activity of cathepsin B in gastrocnemius and soleus (Figure 4.16). Clenbuterol administration increased the total activity of cathepsin B in both gastrocnemius and EDL. In gastrocnemius, clenbuterol restored partially the denervation caused increase in enzyme activity.

On the whole, the effect of denervation on cathepsin B activity in adult rats was significant in both EDL and soleus. However, the effect on gastrocnemius developed slowly. On the fourth day after denervation, no effect of denervation in gastrocnemius was observed. In young rats, increased cathepsin B activity was found only in gastrocnemius and soleus.

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	P<0.005	P<0.05
	Total	N.S.	P<0.005	P<0.05
EDL	Specific	P<0.001	N.S.	P<0.01
	Total	P<0.001	P<0.025	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	P<0.001	P<0.025	N.S.

Figure 4.15 Cathepsin B activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)
Spe.Act.
Total Act.
EDL
Soleus

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	N.S.	P<0.005
	Total	N.S.	P<0.005	N.S.
EDL	Specific	N.S.	N.S.	N.S.
	Total	N.S.	P<0.005	N.S.
Soleus	Specific	P<0.025	N.S.	N.S.
	Total	N.S.	N.S.	N.S.

Figure 4.16 Cathepsin B activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (mU/mg Protein)
Total Activity (mU/Muscle)

Effect of clenbuterol treatment was much more complicated than that of denervation. For gastrocnemius, acute treatment caused lower activity only in control muscle while the one week treatment caused higher activity only in the denervated muscle. In young rats, clenbuterol treatment restored the denervation induced rise of cathepsin B activity. In EDL, clenbuterol treatment reversed the effect elicited by denervation within the first week of treatment but this effect could not be obtained after two weeks of treatment. This effect was not observed in the young rats. In soleus of adult rats, clenbuterol treatment elevated the cathepsin B activity in the control muscle after four days of treatment but decreased this activity in the control muscle after two weeks of clenbuterol treatment. No effect of clenbuterol was found in denervated soleus. In young rats, clenbuterol treatment reduced the denervation caused increase in the activity of this enzyme. However, the result was not significant statistically due to the large errors in the result.

Activity of another lysosomal enzyme, acid phosphatase, was also determined. This can provide more information about the involvement of lysosome in the treatment.

The time course of denervation on this enzyme is shown in Figure 4.17. In gastrocnemius, denervation had no significant effect on the specific activity for a long time. After four weeks, acid phosphatase activity decreased its activity. The activity of this enzyme increased with time

Figure 4.17 Acid phosphatase activities in muscles of rats as a time course of denervation. Method of denervation has been described in Table 4.1. For a muscle, columns with same letters at their top are not significantly different.

Gastrocnemius
EDL
Soleus
control
1 week
2 weeks
4 weeks
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
50
40
30
20
10
0
6000
4000
2000
80
60
400
300
200
100
75
25
h h
h
j
n
o
p
q
k
l
j
h
n
o
p
k
j
h
h
no
n
o
p

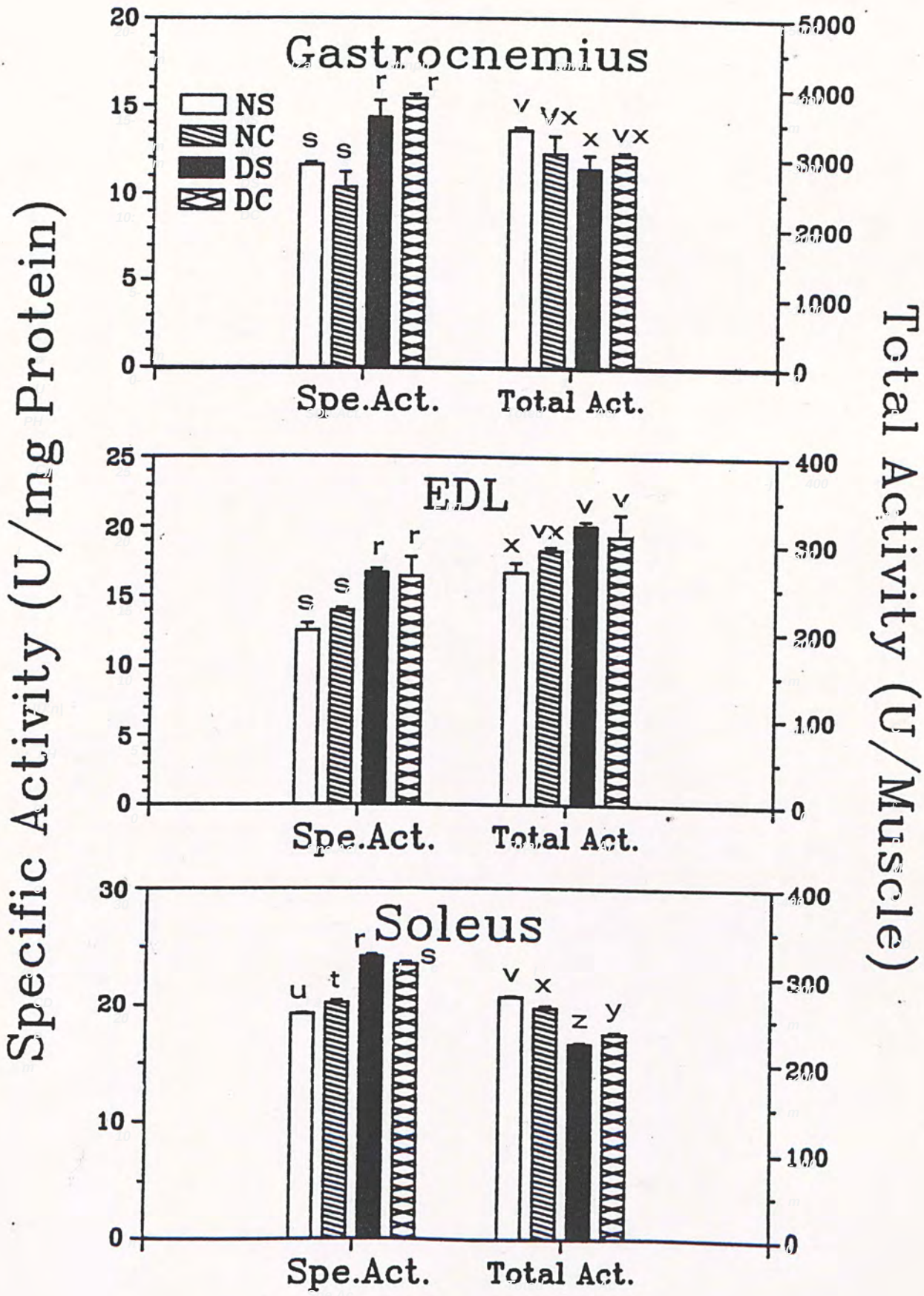
after denervation in EDL and soleus. The increase in soleus stopped two weeks after denervation. The fact that total activity in gastrocnemius increased one week after denervation may be a reflection of the relative high protein concentration in the denervated rat muscle homogenate. The decrease in total activity in all the muscles was caused by the denervation induced reduction in muscle weight.

Acid phosphatase activity in muscles of acutely treated rats shown in Figure 4.18. Denervation increased the specific activity of all the muscles examined significantly. Clenbuterol exerted significant effect only in soleus specific acid phosphatase activity. In control muscles clenbuterol increased this activity but it decreased this activity in denervated muscles. Clenbuterol administration reversed the denervation induced effect. The total activity was elevated in EDL and lowered in soleus by denervation. Significant effect of clenbuterol treatment was found only in soleus. The change in total activity was opposite to that in specific activity in soleus. The total activity may be a reflection of the change in muscle weight as the unit activity (activity per gram muscle) was almost similar.

Figure 4.19 shows acid phosphatase activities in rats subjected to long term treatment. For specific activity, significantly increase activity was found in all the muscles probed after denervation. Clenbuterol treatment lowered the specific activity in denervated gastrocnemius and EDL significantly. Clenbuterol treatment also decreased this

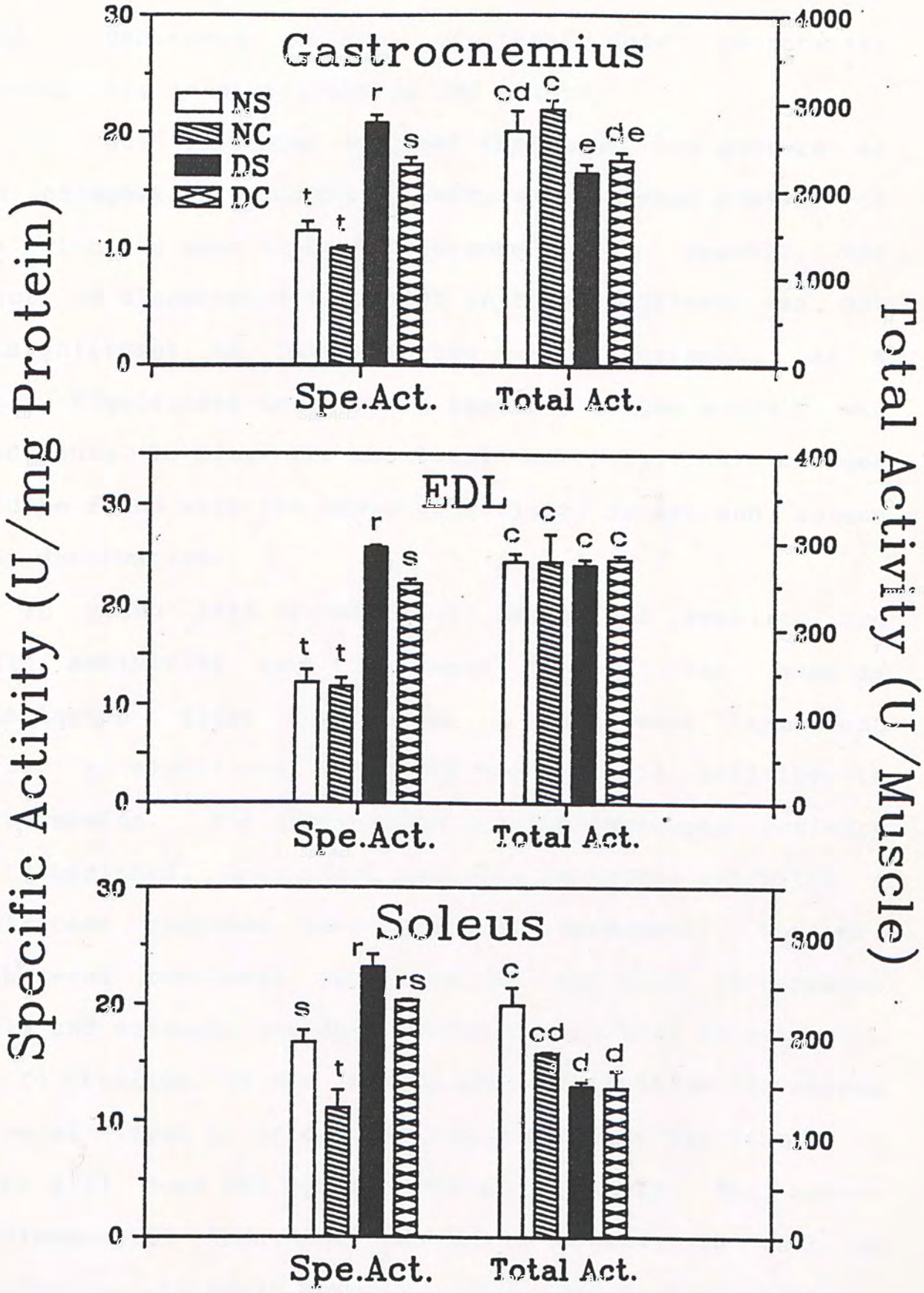
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	N.S.	N.S.
	Total	P<0.005	N.S.	N.S.
EDL	Specific	P<0.005	N.S.	N.S.
	Total	P<0.050	N.S.	N.S.
Soleus	Specific	P<0.005	N.S.	P<0.005
	Total	P<0.001	N.S.	P<0.001

Figure 4.18 Acid phosphatase activities in muscles of rats with acute denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	P<0.005	N.S.
	Total	P<0.005	N.S.	N.S.
EDL	Specific	P<0.001	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	P<0.005	P<0.025	N.S.
	Total	P<0.01	N.S.	N.S.

Figure 4.19 Acid phosphatase activities in muscles of rats with chronic denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

activity in the normal muscles. In soleus, the effect of clenbuterol was significant and no specific effect was found. Decreased activity of total acid phosphatase occurred only in gastrocnemius and soleus.

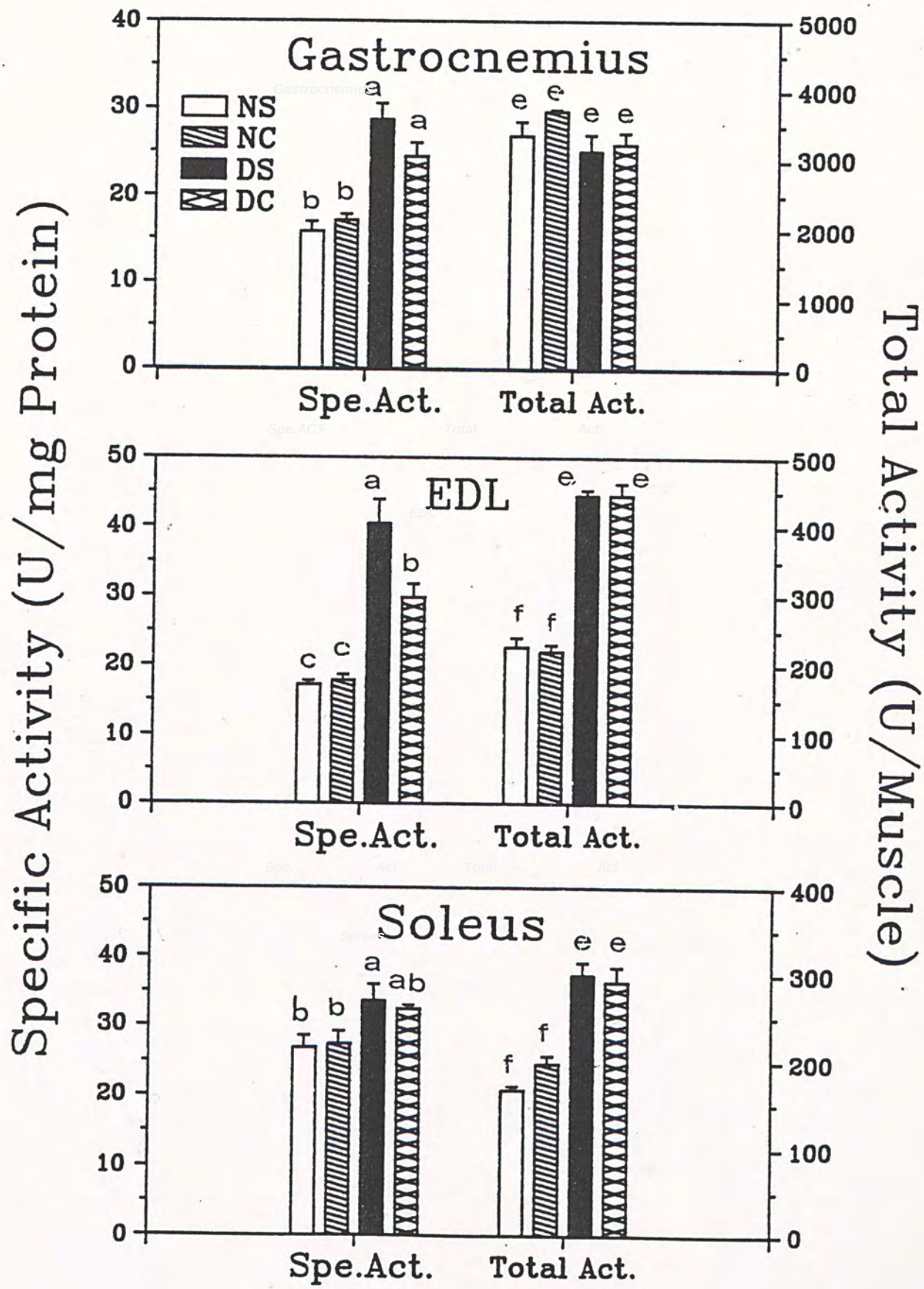
In adult rats with one week treatment, the pattern of acid phosphatase specific activity was somewhat similar to that with two week treatment (Figure 4.20). However, the effect of clenbuterol treatment in this experiment was not as significant as that with two week treatment. As a result, significant decrease in specific enzyme activity was found only in EDL. For the total activity, all changes could be found were the elevated activity in EDL and soleus after denervation.

In young rats (Figure 4.21), both the specific and total activities were increased in all the muscles investigated after denervation. Clenbuterol treatment exerted a significant effect on the specific activity in gastrocnemius. The denervation induced increased activity was diminished. Only total activity in soleus exhibited a significant response to clenbuterol treatment. However, clenbuterol treatment increased the activity in control muscle and enhanced the denervation caused rise in activity.

To conclude, it was obvious that denervation developed increased level of acid phosphatase although the result in Figure 4.17 does not agree to this completely. The effect of clenbuterol treatment was not as obvious as that of denervation. In adult animals, after four days of treatment (acute treatment), only soleus showed significant response

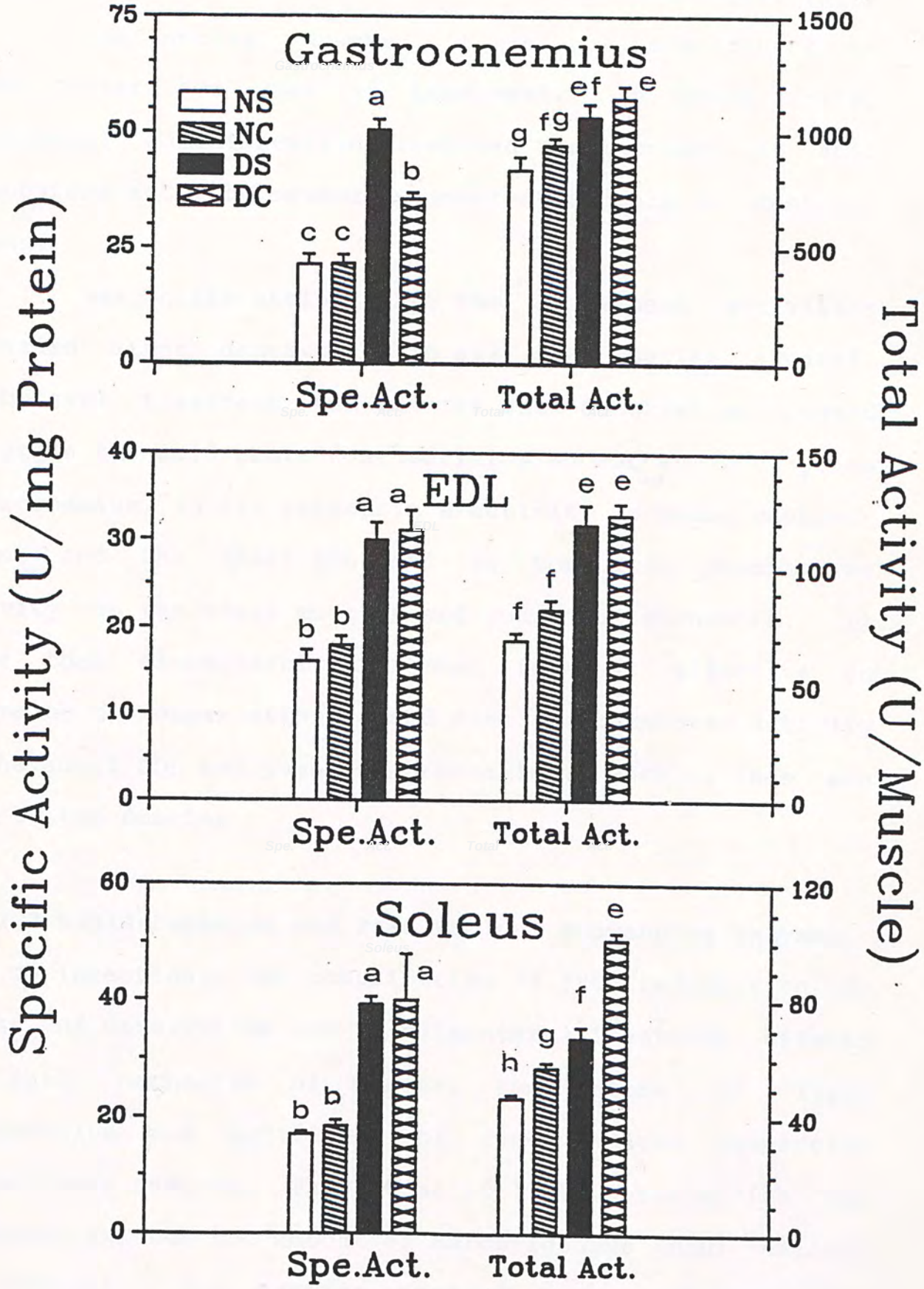
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
EDL	Specific	P<0.001	P<0.05	P<0.05
	Total	P<0.001	N.S.	N.S.
Soleus	Specific	P<0.01	N.S.	N.S.
	Total	P<0.001	N.S.	N.S.

Figure 4.20 Acid phosphatase activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	P<0.01	P<0.01
	Total	P<0.005	N.S.	N.S.
EDL	Specific	P<0.001	N.S.	N.S.
	Total	P<0.001	N.S.	N.S.
Soleus	Specific	P<0.001	N.S.	N.S.
	Total	P<0.001	P<0.001	P<0.005

Figure 4.21 Acid phosphatase activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Spe.Act.
Total Act.
EDL
Soleus
75
50
25
0
1500
1000
500
40
30
20
10
150
100
60
120
80
c c a b g fg ef e
b b a a f f e e
b b a a h g f e

to the clenbuterol treatment. Clenbuterol reversed the change caused by denervation. This effect developed later in all the muscles. However, it was not significant in soleus after two week of treatment. In young rats, clenbuterol administration restored the increase in acid phosphatase activity caused by denervation only in gastrocnemius.

It was quite obvious that the proteinase activities increased after denervation in all the muscles studied. Clenbuterol treatment can reverse the denervation caused effect in the acid proteinase activity on EDL and the young gastrocnemius, in the cathepsin B activity on young gastrocnemius and the adult EDL, and in the acid phosphatase activity on the adult muscles and young gastrocnemius. It seems that clenbuterol treatment is most effective in reversing the denervation caused rise in proteinase activity in the adult EDL and young gastrocnemius. Both of them are fast twitch muscles.

4.3.2.3 Lipid Peroxide and Free Radical Scavenging Enzymes

To investigate the contribution of free radicals on the damage of denervation and if clenbuterol treatment effects on this mechanism of damage, the degree of lipid peroxidation and activities of free radical scavenging enzymes were assayed. The extent of lipid peroxidation was evaluated through the amount of malonaldehyde (MAD) existed in muscles. The enzymes assayed were catalase and superoxide dismutase (SOD).

Table 4.14 Malonaldehyde content in muscles and liver of adult rats with hind limb denervatin and clenbuterol treatment

	Gastrocnemius		Liver	
NS	0.141 ± 0.011[a]	D: N.S.	0.244 ± 0.026[b]	D: N.S.
NC	0.135 ± 0.000[a]	C: N.S.	0.371 ± 0.041[a]	C: P<0.025
DS	0.114 ± 0.013[a]	D*C:N.S.	0.386 ± 0.012[a]	D*C :N.S.
DC	0.128 ± 0.005[a]		0.328 ± 0.030[ab]	

	EDL		Soleus	
NS	0.123 ± 0.004[a]	D: N.S.	0.109 ± 0.006[a]	D: N.S.
NC	0.123 ± 0.006[a]	C: N.S.	0.120 ± 0.008[a]	C: N.S.
DS	0.112 ± 0.011[a]	D*C:N.S.	0.113 ± 0.009[a]	D*C: N.S.
DC	0.114 ± 0.008[a]		0.111 ± 0.010[a]	

Values shown here are absorbance at 532 nm for muscles and the difference of absobance at 532 nm and 520 nm after the homogenates reacting with thiobarbitaric acid.
EDL : Extensor digitorum longus
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

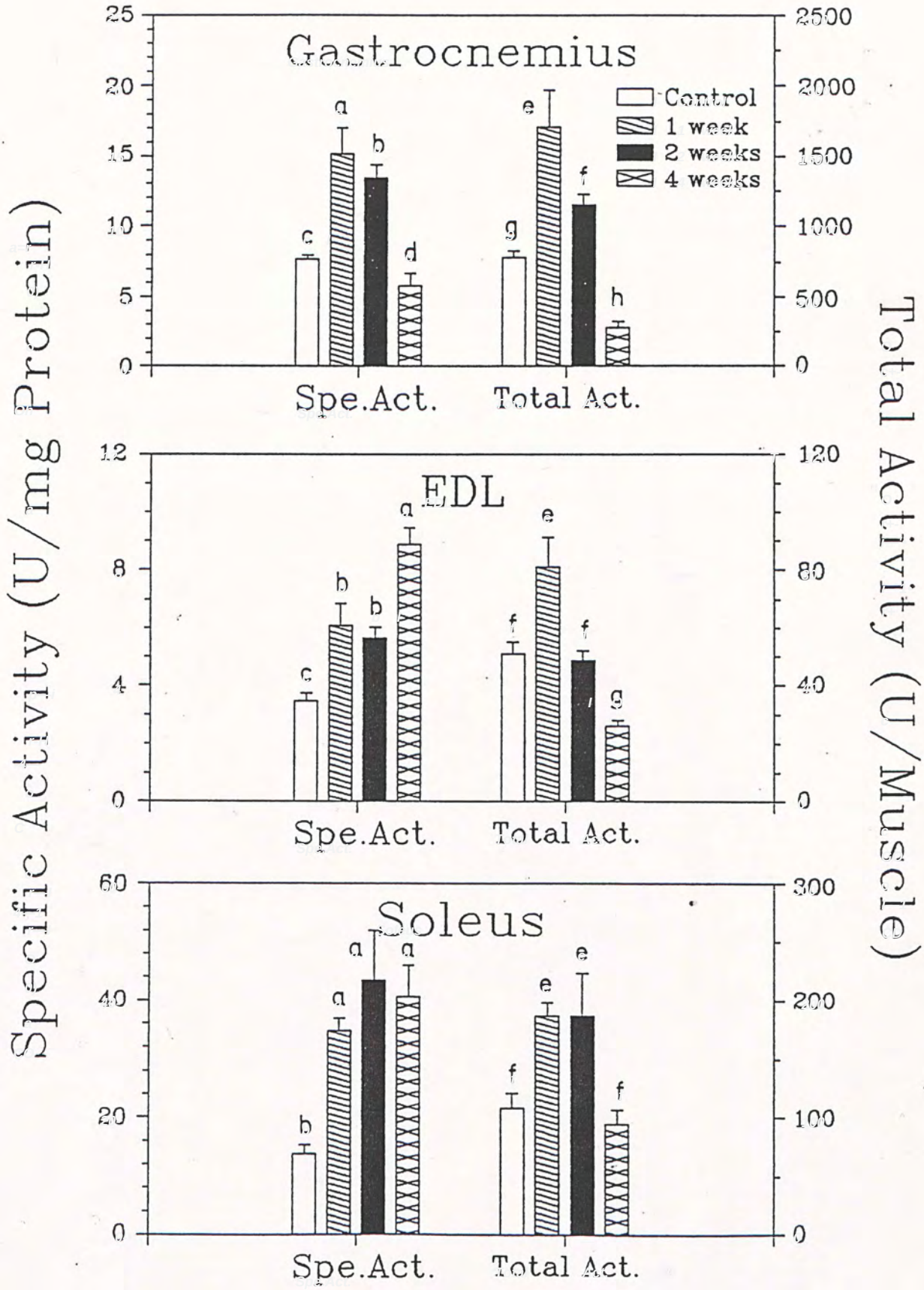
Amount of MAD in muscle homogenates and in liver was determined only in the adult rats with one week of treatment. The amount of MAD is shown in Table 4.14 as the value of absorbance at 532 nm after the reaction of muscle homogenate with thiobarbituric acid (please refer to Chapter 2 for the details of the method). In all the muscles investigated, the absorbance value showed a trend of decrease in the denervated muscle but this was not significant statistically. Both denervation and clenbuterol treatment had no significant effect on lipid peroxide in muscle. However, clenbuterol treatment caused significant rise in amount of MAD in liver of control animals.

The first enzyme to be assayed was catalase. It is responsible for the degradation of H_2O_2 which is produced by the dismutation of oxygen radical originated in many cellular processes.

The time course of denervation effect on the muscles is shown in Figure 4.22. In gastrocnemius, the enzyme activity increased by almost two fold one week after the operation. The enzyme level then decreased gradually. In EDL and soleus, the enzyme activity was also increase by almost two fold after one week but the activity was maintained at this level for a period more than four weeks. The decrease in the total activity was a consequence of the much lowered muscle weight after the operation for a certain time.

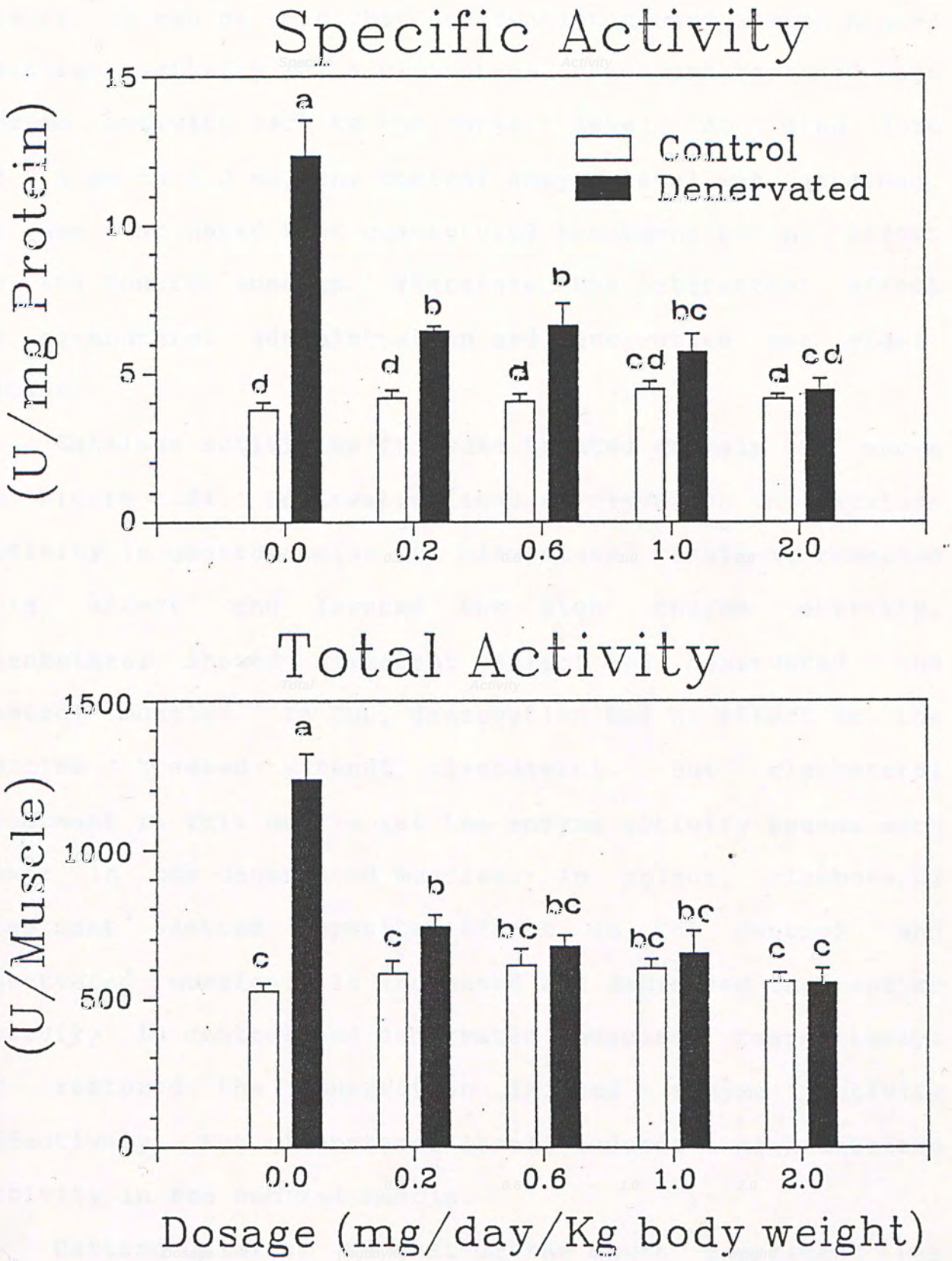
The dose effect of clenbuterol treatment on gastroc-

Figure 4.22 Catalase activities in muscles of rats as a time course of denervation. Method of denervation has been described in Table 4.1. For a muscle, columns with same letters at their top are not significantly different.


Gastrocnemius
Control
1 week
2 weeks
4 weeks
Spe.Act.
Total Act.
EDL
Soleus
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

	D	C	D*C
Specific activity	P<0.001	P<0.001	P<0.001
Total activity	P<0.001	P<0.001	P<0.001

Figure 4.23 Dose effect of clenbuterol on catalase activities of denervated and control gastrocnemius. Dose of clenbuterol used was in unit of mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.


Specific Activity
(U/mg Protein)
15
10
5
0
Control
Denervated
a
d
d
b
a
b
cd
bc
d
cd
0.0
0.2
0.6
1.0
2.0
Total Activity
(U/Muscle)
1500
1000
500
0
a
c
c
b
bc
bc
bc
bc
c
c
0.0
0.2
0.6
1.0
2.0
Dosage (mg/day/Kg body weight)

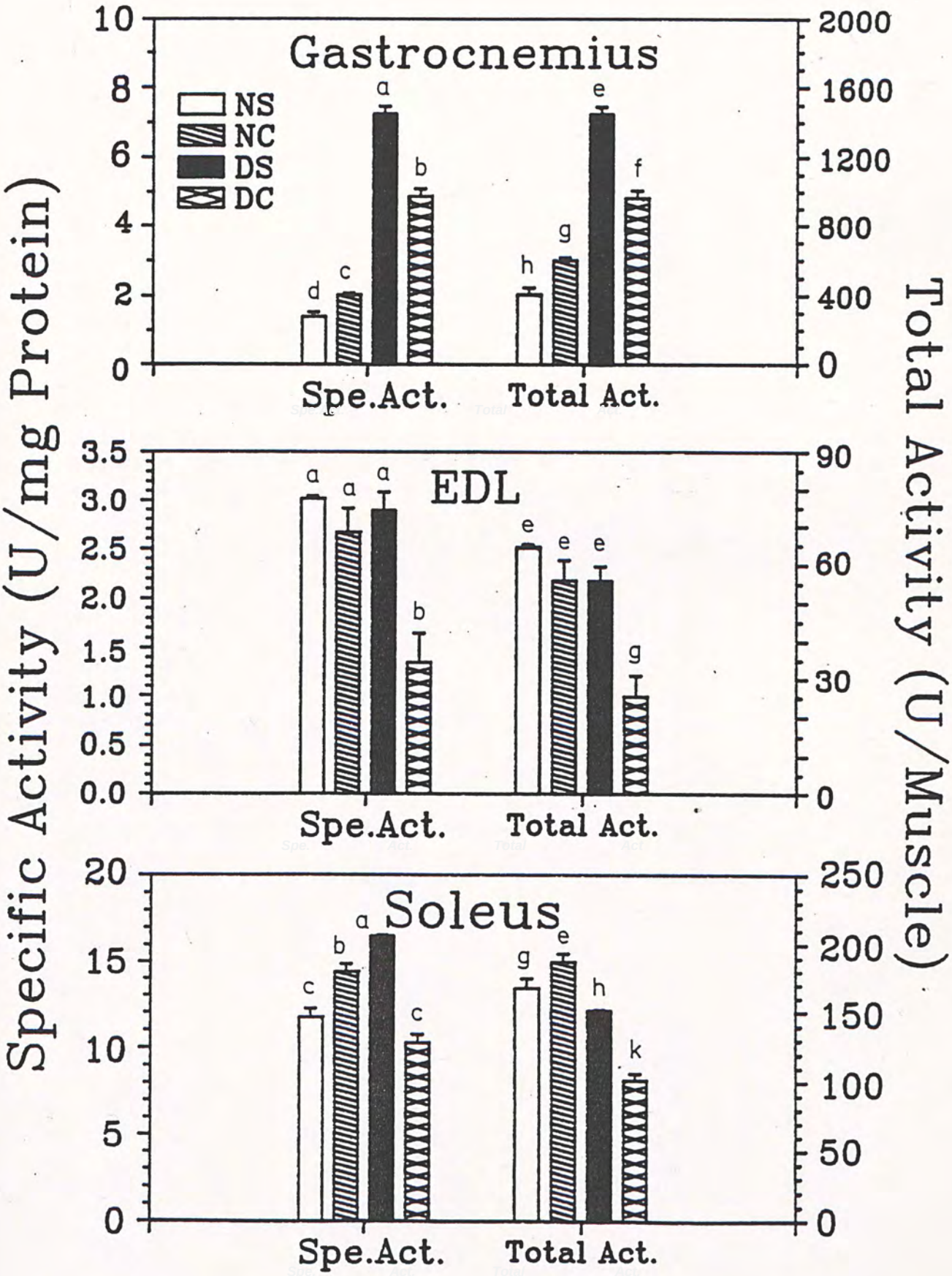
nemius was also investigated (Figure 4.23). From the figure, it can be seen that denervation caused a much higher catalase activity while clenbuterol treatment restored the enzyme activity back to the control level. At a drug dose of 0.6 mg to 1.0 mg, the control enzyme level was attained. It was also noted that clenbuterol treatment had no effect on the control muscles. Therefore, the interaction effect of clenbuterol administration and denervation was significant.

Catalase activities in acute treated animals are shown in Figure 4.24. Denervation induced elevation in catalase activity in gastrocnemius but clenbuterol treatment reversed this effect and lowered the high enzyme activity. Clenbuterol showed different effect on denervated and control muscles. In EDL, denervation had no effect on the muscles treated without clenbuterol. But clenbuterol treatment in this muscle let the enzyme activity become much lower in the denervated muscles. In soleus, clenbuterol treatment exerted opposite effect on the control and denervated muscles. It increased and decreased the enzyme activity in control and denervated muscles, respectively. It restored the denervation induced enzyme activity effectively. But clenbuterol itself induced a high catalase activity in the control muscle.

Pattern similar to that of the acute experiment was found in gastrocnemius of rats subjected to long term treatment (Figure 4.25). In EDL and soleus, denervation

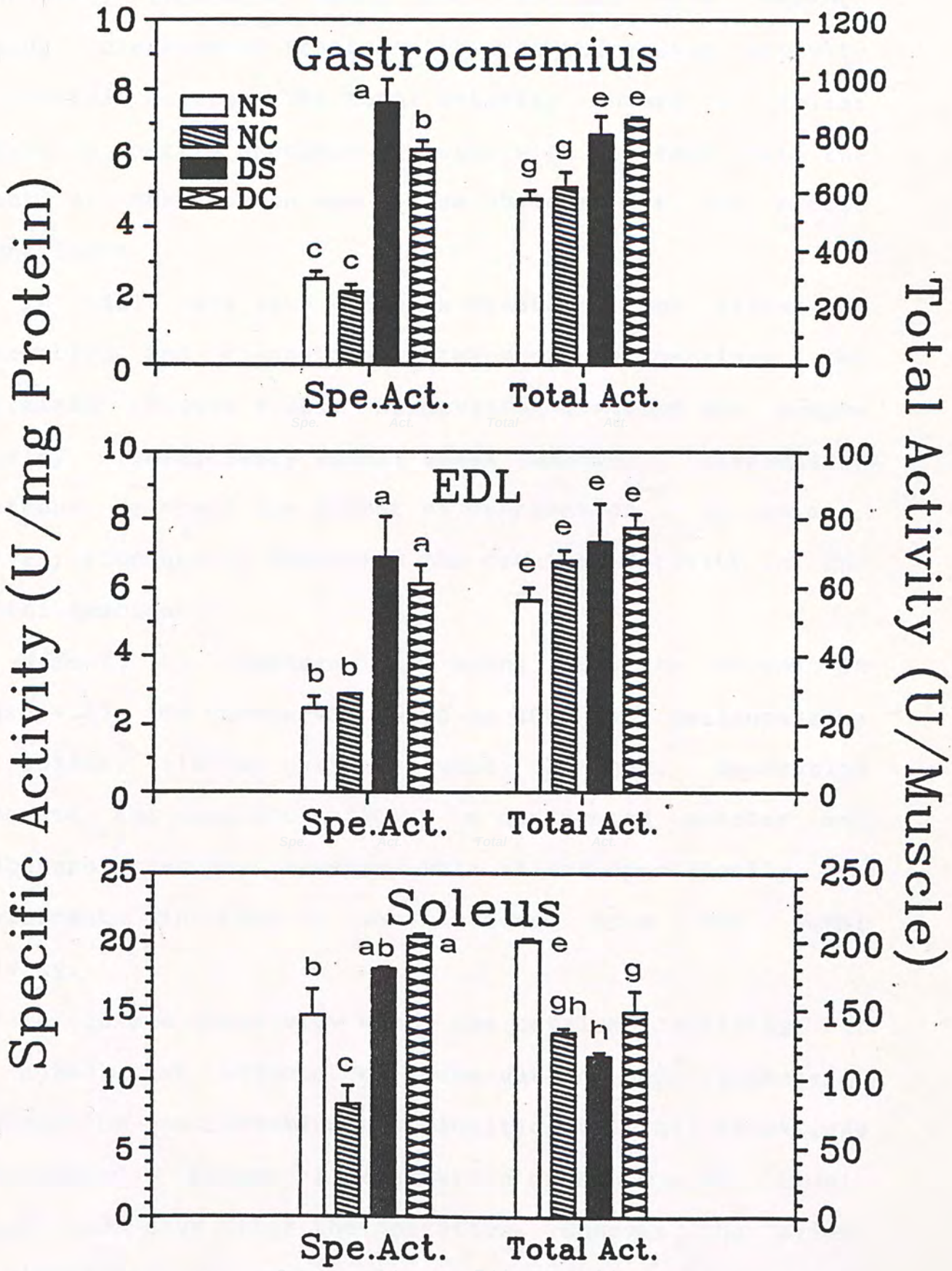
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	P<0.005	P<0.001
	Total	P<0.001	P<0.01	P<0.001
EDL	Specific	P<0.01	P<0.005	N.S.
	Total	P<0.005	P<0.005	P<0.025
Soleus	Specific	N.S.	P<0.005	P<0.001
	Total	P<0.001	P<0.025	P<0.001

Figure 4.24 Catalase activities in muscles of rats with acute denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
EDL
Soleus
NS
NC
DS
DC
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
0
2
4
6
8
10
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
5
10
15
20
400
800
1200
1600
2000
30
60
90
50
100
150
200
250
a
b
c
d
e
f
g
h
k

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	P<0.05	N.S.
	Total	P<0.001	N.S.	N.S.
EDL	Specific	P<0.005	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	P<0.005	N.S.	P<0.025
	Total	P<0.025	N.S.	P<0.005

Figure 4.25 Catalase activities in muscles of rats with chronic denervation and clenbuterol treatment. Dose of clenbuterol used was 0.2 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
EDL
Soleus
NS
NC
DS
DC
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Spe.Act.
Total Act.
0
2
4
6
8
10
0
200
400
600
800
1000
1200
0
20
40
60
80
100
0
5
10
15
20
25
0
50
100
150
200
250

caused significant increased catalase activity and clenbuterol treatment could not reversed this effect. Moreover, clenbuterol treatment lowered the enzyme activity in control soleus. The total activity showed a similar pattern to that in specific activity with the fact that the effect of denervation was not so obvious for the muscle weight loss.

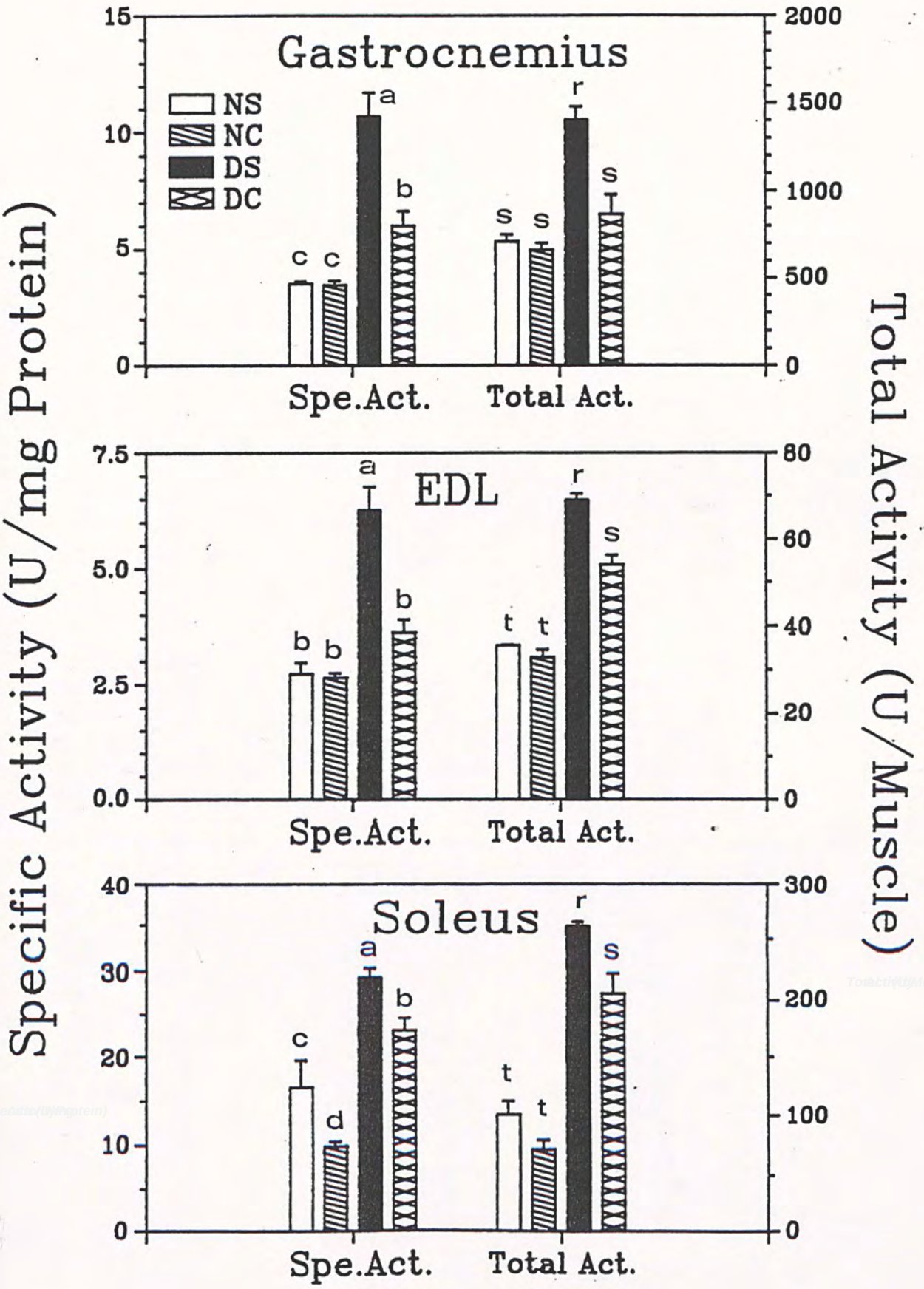
In adult rats with one week treatment, the effect of denervation and clenbuterol treatment on catalase was determined (Figure 4.26). Denervation elevated the enzyme activity significantly in all three muscles. Clenbuterol treatment reversed the effect of denervation. In soleus, however, clenbuterol decreased the catalase activity in the control muscles.

Effect of treatment in young rats is shown in Figure 4.27. No change was found in EDL. In gastrocnemius and soleus, similar patterns were obtained. Denervtion increased the specific activity in denervated muscles and clenbuterol treatment reversed this effect specifically. No significant information was obtained from the total activity.

To take a whole view about the catalase activity, it was noted that effects of denervation and clenbuterol treatment on gastrocnemius was significant in all situations investigated. Effect of denervation on EDL was not significant four days after the operation. However, the effect was significant one week or longer after the operation. In EDL of the young rats, there was still no significant

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	P<0.005	P<0.005
	Total	P<0.001	P<0.005	P<0.01
EDL	Specific	P<0.001	P<0.005	P<0.005
	Total	P<0.001	P<0.001	P<0.01
Soleus	Specific	P<0.001	P<0.01	N.S.
	Total	P<0.001	P<0.005	N.S.

Figure 4.26 Catalase activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
EDL
Soleus
NS
NC
DS
DC
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Spe.Act.
Total Act.
15
10
5
0
2000
1500
1000
500
7.5
5.0
2.5
0.0
80
60
40
20
40
30
20
10
300
200
100

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.001	P<0.001	P<0.001
	Total	P<0.05	P<0.005	P<0.025
EDL	Specific	N.S.	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	P<0.025	P<0.005	P<0.05
	Total	N.S.	P<0.05	N.S.

Figure 4.27 Catalase activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

Specific Activity (U/mg Protein)
Total Activity (U/mg Protein)
Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.

denervation effect one week after the operation. Clenbuterol decreased catalase activity in the denervated EDL when the treatment period was very long. In soleus, the effects of denervation and clenbuterol treatment was significant four days and one week after the treatment. But these effects disappeared after two week treatment. The effects were also significant in the young rats.

Table 4.15 Catalase activity in hemolysate of rats with denervation in hind limbs and clenbuterol treatment

	Volume Activity		Specific Activity	
NS	64.03 ± 8.69^{b}	D:P<0.05	7.997 ± 0.815^{a}	D:P<0.05
NC	70.88 ± 4.30^{ab}	C: N.S.	5.710 ± 0.509^{b}	C:P=0.06
DS	92.53 ± 10.25^{a}	D*C:N.S.	5.581 ± 0.384^{b}	D*C: P=0.06
DC	81.23 ± 6.78^{ab}		5.559 ± 0.337^{b}	

Values are expressed as enzyme unit/ml hemolysate in volume activity and enzyme unit/mg protein in specific activity. Hemolysate was prepared as that descibed by Matkovics et al. (1982).
NS : Normal, saline-treated; NC : Normal, clenbuterol-treated; DS : Denervated, saline-treated; DC : Denervated, clenbuterol-treated. Methods of denervation and clenbuterol administration has been described in Table 4.1 and 4.2.
D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant
Values with same superscript letters are not significantly different.

Catalase activity in hemolysate was also determined (Table 4.15). It was found that only denervation exerted significant effect on the enzyme activity. As in the muscle, catalase activity was elevated by denervation. This may showed that the denervation effect on catalase activity was not specific to the muscle.

Another free radical scavenging enzyme studied was SOD. There are two types of SOD, CuZnSOD and MnSOD. Both of them were measured in our experiment. The total SOD activity was also determined. Figure 4.28 shows the change of MnSOD in adult rats. No significant effect was found in specific activity of all the muscles studied. Denervation exerted its effect only in the total activity in gastrocnemius and soleus. It decreased the enzyme activity in gastrocnemius while increased the enzyme activity in soleus.

In young rats, MnSOD was also mostly unaffected by the two factors investigated. Significant difference was found only in the total activity in gastrocnemius (Figure 4.29).

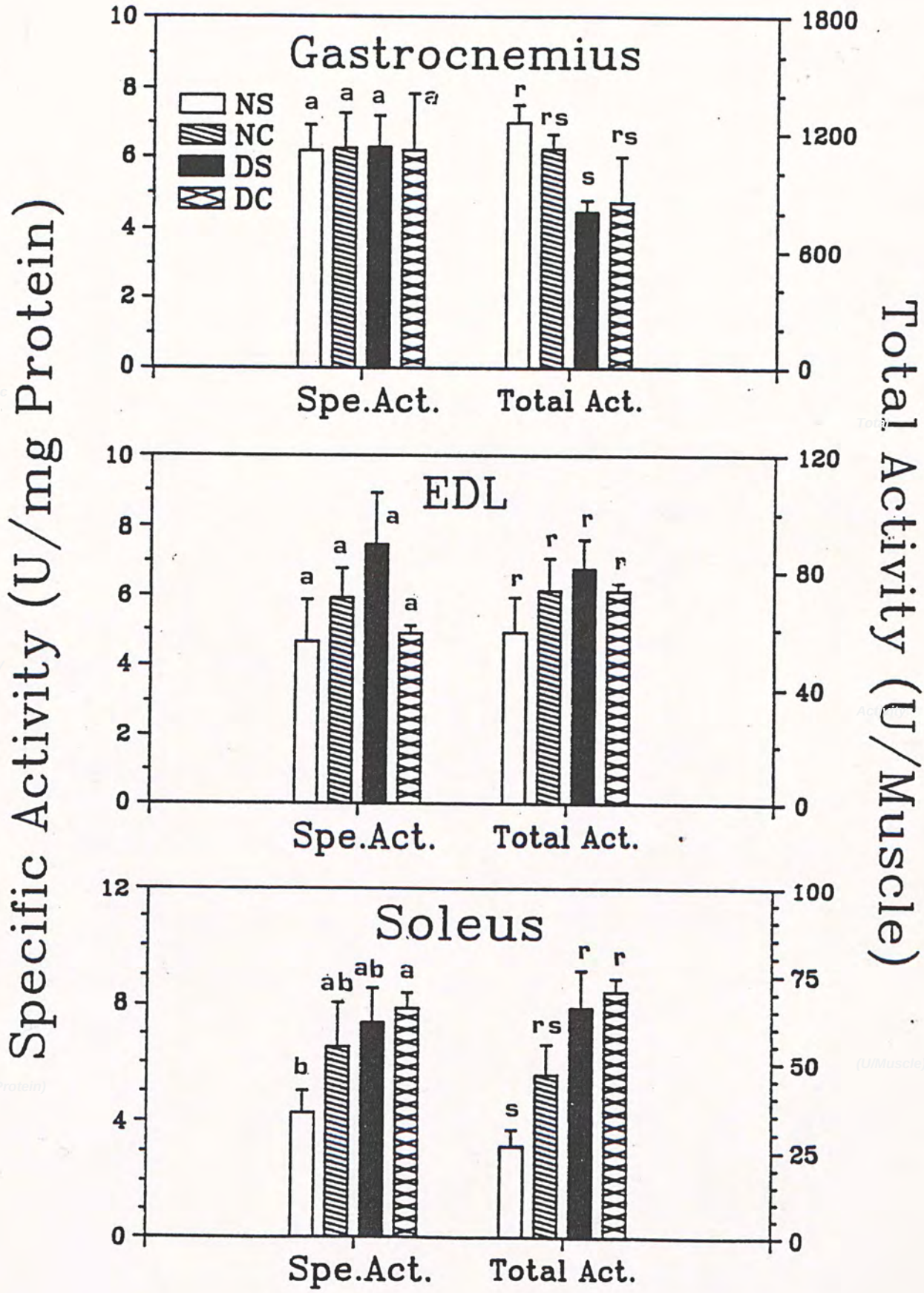
As a conclusion, it seems that MnSOD activity was not affected by denervation and clenbuterol treatment.

Activity of CuZnSOD in adult rat muscles is shown in Figure 4.30. A picture similar to the MnSOD activity was found. No significant change in specific activity was found. Denervation caused rise in total catalase activity in soleus and decrease in the total activity in gastrocnemius.

Figure 4.31 shows the effect of denervation and

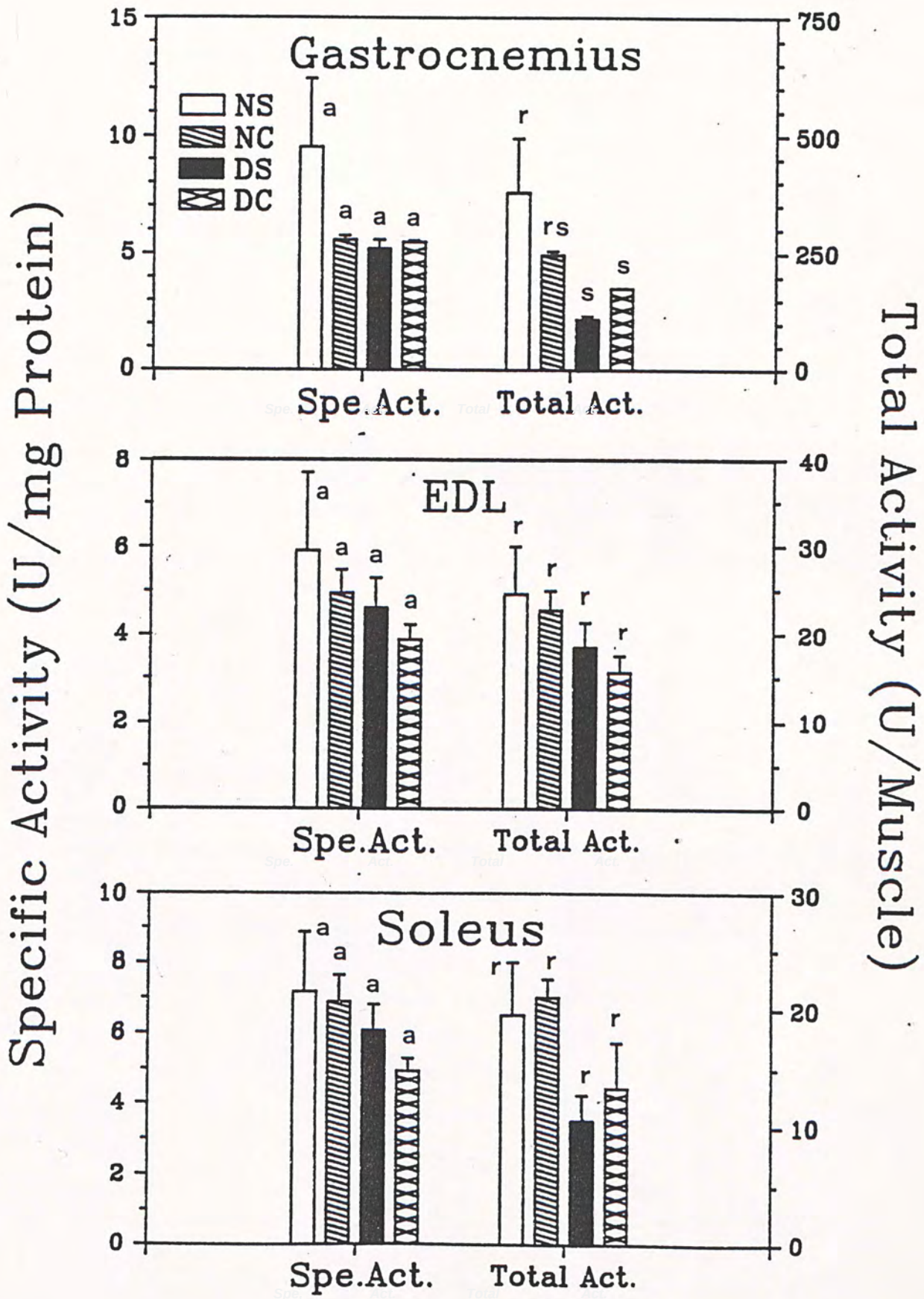
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	N.S.
	Total	P<0.05	N.S.	N.S.
EDL	Specific	N.S.	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	P<0.005	N.S.	N.S.

Figure 4.28 MnSOD activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	N.S.
	Total	P<0.025	N.S.	N.S.
EDL	Specific	N.S.	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	N.S.	N.S.	N.S.

Figure 4.29 MnSOD activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Soleus
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

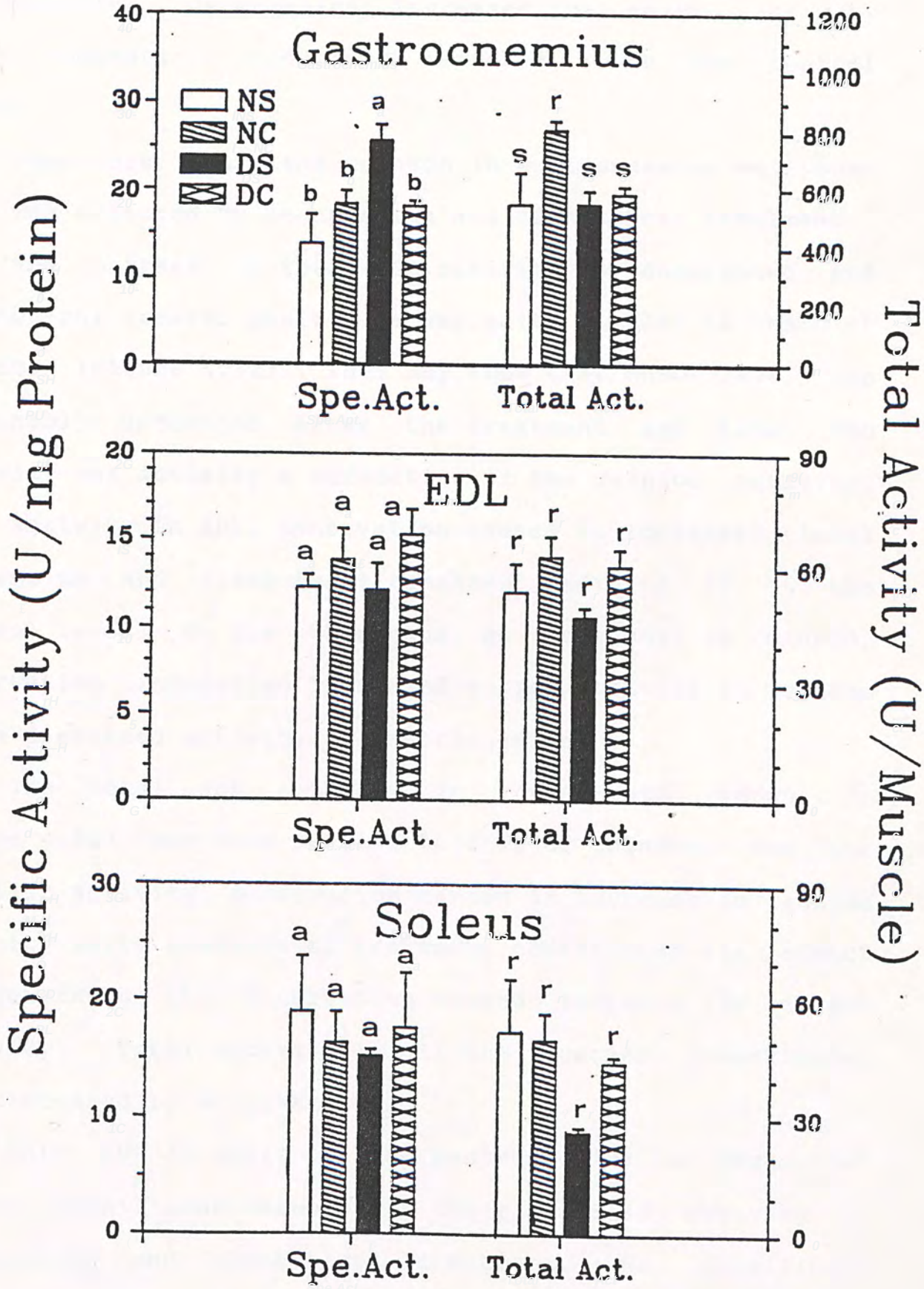
Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	N.S.
	Total	P<0.05	N.S.	N.S.
EDL	Specific	N.S.	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	P<0.05	N.S.	N.S.

Figure 4.30 CuZnSOD activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Soleus

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.025	N.S.	P<0.025
	Total	N.S.	P<0.05	N.S.
EDL	Specific	N.S.	N.S.	N.S.
	Total	N.S.	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	N.S.	N.S.	N.S.

Figure 4.31 CuZnSOD activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Spe.Act.
Total Act.
EDL
Spe.Act.
Total Act.
Soleus
Spe.Act.
Total Act.
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)

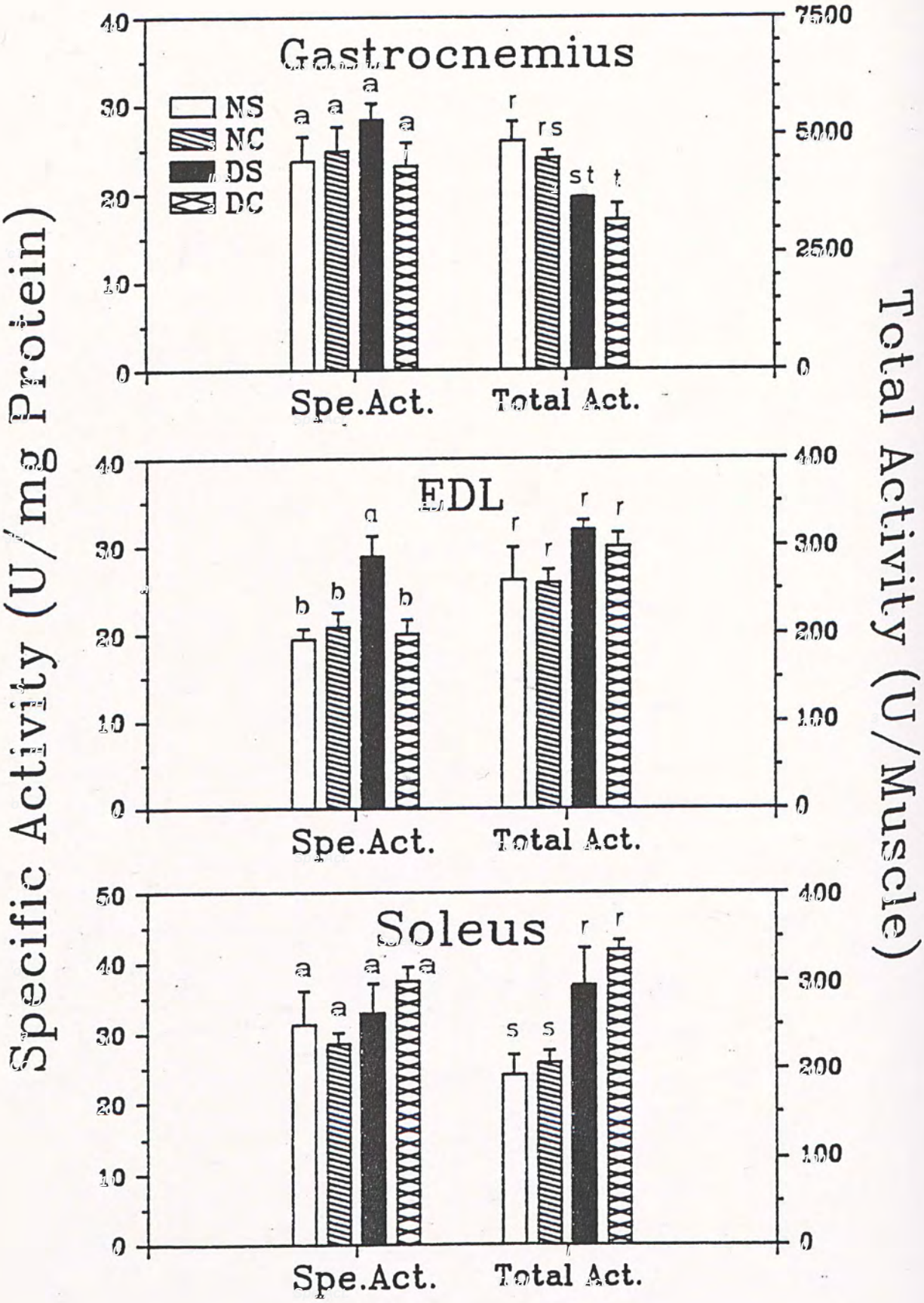
clenbuterol treatment on the CuZnSOD activity in young rats. In EDL and soleus, no significant effect was observed. In gastrocnemius, denervation increased the enzyme activity while clenbuterol treatment restored it to the control level.

Therefore, only the CuZnSOD in gastrocnemius of young rats was affected by denervation and clenbuterol treatment.

The pattern of total SOD activity in denervated and clenbuterol treated adult rats was quite similar to that of CuZnSOD (Figure 4.32). This may show that MnSOD level was reasonably unchanged after the treatment and total SOD activity was actually a reflection of the CuZnSOD activity. For activity in EDL, denervation caused an increased level of enzyme and clenbuterol treatment restored it to the control level. On the other hand, as the result of CuZnSOD, denervation induced an increased enzyme activity in soleus and a decreased activity in gastrocnemius.

The total SOD activity in young rats (shown in Figure 4.33) was also similar to that of CuZnSOD. For the specific activity, denervation caused an increase in enzyme activity while clenbuterol treatment contributed its effect by reversing the denervation caused increase in enzyme activity. Total activity in all the muscles investigated was increased by denervation.

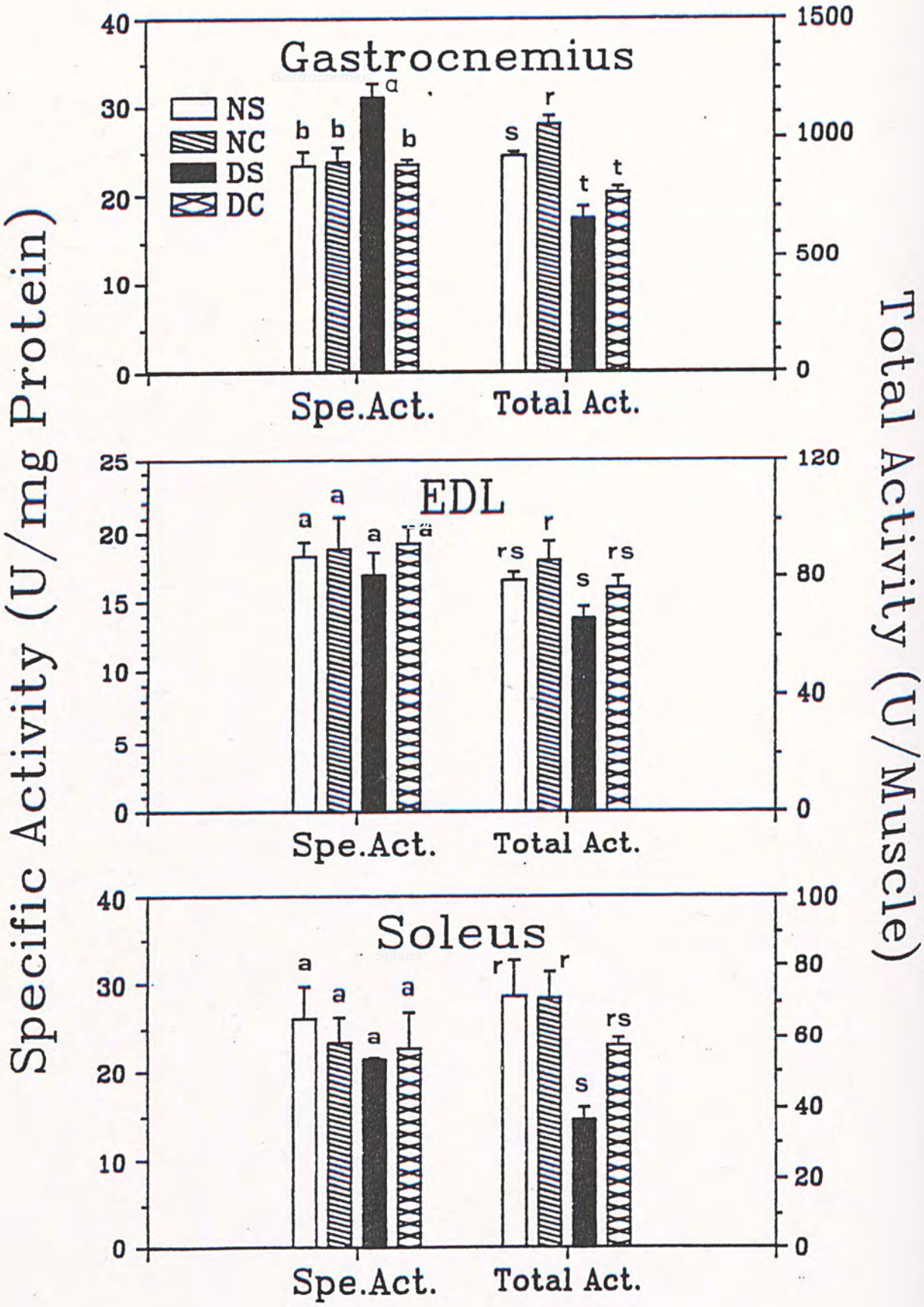
Only EDL in adult rat and gastrocnemius in young rat showed significant response in their specific activity to denervation and clenbuterol treatment. No significant effect was found in the specific activity of soleus.


Gastrocnemius
NS
NC
DS
DC
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Spe.Act.
Total Act.
EDL
Soleus
0
10
20
30
40
50
100
200
300
400
2500
5000
7500

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	N.S.	N.S.	N.S.
	Total	P<0.005	N.S.	N.S.
EDL	Specific	P<0.05	N.S.	P<0.025
	Total	P=0.059	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	P<0.005	N.S.	N.S.

Figure 4.32 Total SOD activities in muscles of adult rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.

NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated


Gastrocnemius
NS
NC
DS
DC
Specific Activity (U/mg Protein)
Total Activity (U/Muscle)
Spe.Act.
Total Act.
EDL
Soleus

Muscles	Activity	D	C	D*C
Gastrocnemius	Specific	P<0.05	P<0.05	P<0.05
	Total	P<0.001	P<0.01	N.S.
EDL	Specific	N.S.	N.S.	N.S.
	Total	P<0.05	N.S.	N.S.
Soleus	Specific	N.S.	N.S.	N.S.
	Total	P<0.025	N.S.	N.S.

Figure 4.33 Total SOD activities in muscles of young rats with one week denervation and clenbuterol treatment. Dose of clenbuterol used was 0.6 mg/kg body-weight per day. Methods of denervation and clenbuterol administration have been described in Table 4.1 and 4.2. The table above shows the significant contribution of the different factors studied. D : Significance of denervation caused effect; C : Significance of clenbuterol treatment caused effect; D*C : Significance of the interaction effect of denervation and clenbuterol treatment; N.S. : non-significant. For a muscle, columns with same letters at their top are not significantly different.
NS : Normal, saline-treated
NC : Normal, clenbuterol-treated
DS : Denervated, saline-treated
DC : Denervated, clenbuterol-treated

On the whole, it was found that the level of lipid peroxide was not changed with either denervation or clenbuterol administration. For catalase activity, denervation caused significant increase and clenbuterol treatment reversed this effect in gastrocnemius, soleus, and adult EDL. There was no change in MnSOD activity after the treatment. The treatment had its effect on the total SOD and CuZnSOD activities in only the young gastrocnemius and adult EDL. This is fairly consistent with the result of the proteinase activity.

4.4 Conclusion and Discussion

Denervation of skeletal muscle is resulted by the interruption of the normal activity of the motor nerve to a skeletal muscle caused either by disease or severe mechanical injury. Usually the muscle will be reinnervated either by the regenerated fiber or a nearby fiber (McComas, 1977). However, these processes need time and in the period reinnervation is going on the denervated muscle undergoes atrophy. After a few weeks of atrophy, much of the mass of the muscle is replaced by connective tissue (Sunderland and Ray, 1950). In this situation the muscle can no longer function properly even if reinnervation occurs. Thus, drugs inhibiting or ameliorating the atrophy process in denervated muscles may be helpful.

Clenbuterol, a β_2-adrenergic agonist, has been reported

to ameliorate denervation-induced atrophy in rat soleus muscles (Maltin et al., 1986b). This drug was reported as a growth promoting substance in increasing muscle growth (Baker et al., 1984; Ricks et al., 1984). The growth promoting effect has been attributed to modifications of protein turnover (Emery et al., 1984; Reeds et al., 1986). It has been shown that the ameliorative effects of clenbuterol in denervated rat muscles were due principally to a large increase in protein synthesis. The rest of the observed the protein anabolism was achieved through reduced degradation (Maltin et al., 1987b).

However, besides protein turnover, there are numerous other biochemical changes occurring in denervated muscles. These may also contribute to the atrophy process.

Gross changes in animals under clenbuterol administration gives the most basic information about the treatment. At first, by investigating the time effect, the dose used was according to the result of Reeds et al. (1986) and was 0.2 mg/kg body-weight per day. However, after examining the dose effect of clenbuterol on gross changes such as body weight (Table 4.3), muscle weight (Table 4.8 and Table 4.9) and organ weight (Table 4.5 and Table 4.6), a dose of 0.2 mg/kg body-weight per day was found to be too small to induce a significant effect. As a result, a higher dose (0.6 mg/kg body-weight per day) was used in subsequent experiments in which the age effect was studied.

For body weight, denervation represses its increase in

adult rats but not in young rats. Although clenbuterol administration did not increase the body weight in adult rats, it was obvious that body weight of young rats were increased. Raised body weight gain by treatment with clenbuterol in young rats was also reported by Emery et al. (1984). These show that elevated body weight gain can only be obtained in young rats.

In the organs studied, only liver and kidney respond to the treatment significantly. Denervation increase their weights while clenbuterol administration restored them to the control values. It has been reported that clenbuterol does not affect the weight of kidney and liver after 7 days (Emery et al., 1984) and 15 days (Reeds et al., 1986) treatment. Our result is consistent with these observation. Clenbuterol had no effect on the control rats. Moreover, in our experiment, it was found that clenbuterol restored partially the denervation induced weight increase in these organs. Heart weight has also been reported to be higher in clenbuterol treated rats after 15 days of treatment (Reeds et al., 1986). This effect was not found in our experiment. This may be caused by the shorter treatment period we used.

In denervated muscles, the proteolytic activity increases and thus more amino acids are released. It was also reported that activities of alanine and aspartate aminotransferases increase significantly in denervated muscles and it was suggested that increased utilization of amino acids function as a compensatory metabolic support during denervation atrophy. When amino acids are used as

the energy source, the problem that the amino acid nitrogen must be transferred from muscle to liver happens. The glucose-alanine cycle was proposed (Mallette et al., 1969; Felig et al., 1970). After muscle denervation, more amino acids would have to be transferred to liver. This will enlarge the amino acid pool in liver and probably stimulate protein synthesis in liver. Thus, liver increases its weight. Restoration of liver weight in clenbuterol treated denervated rats may be a result of the lower protein degradation in denervated muscles or a result that the β agonist inhibits the amino acid release in skeletal muscle (Garber et al., 1976).

Muscles in general responded to denervation by lowering their weight. With clenbuterol administration, however, their weight was resumed. In the muscles examined, EDL had no response. Soleus responsed only when the dose was high enough and the period of administration was long enough. Increased weight of soleus and gastrocnemius and the lesser degree of response of EDL after clenbuterol administration had also been reported by other groups (Reeds et al., 1986; Maltin et al., 1987a). The fact that the least effect was found in EDL may reflect that type II fibers has a low response to clenbuterol. This is consistent with the finding that responses in slow twitch oxidative (type I) fibers to clenbuterol were particularly marked (Maltin et al., 1986b). It has also been shown that fast twitch muscle was physiologically insensitive to catecholamine

in vivo (Festoff et al., 1977).

The major difference among various types of fibers is mainly in the energy source or the metabolic patterns. The diverse effect of clenbuterol on different types of muscle may also be significant in muscle metabolism. Moreover, denervation caused changes in metabolism of skeletal muscles. On the other hand, a number of metabolic and physiological processes in skeletal muscle has been reported to be influenced by β-adrenergic stimulation, including glycolysis, glucose uptake, and under some conditions contractility and membrane potential (Drummond et al., 1969; Flatman and Clausen, 1979; Reddy and Engel, 1979; Buur et al., 1982; Richter et al., 1982). Thus, it is quite possible that clenbuterol affect the growth of the muscles through a variety of mechanism. Therefore, it is valuable to assay the effect of clenbuterol on muscles.

For MDH activity, denervation lowered its activity in the muscles examined except in adult gastrocnemius where an increase has been observed. The effect of clenbuterol is mainly opposite to that of denervation with the exception of the chronically treated adult soleus. The decreased MDH activity in denervated muscles agrees to the reported result (Simard et al., 1985). The effect of clenbuterol on denervated muscles might indicate a metabolic basis for the reversal of denervation induced. The diminished effect of chronic clenbuterol treatment on soleus illustrates that there may be a desensitization effect in skeletal muscles after long term administration of the drug. This

desensitization effect has been reported to occur in the central nervous system (Frazer et al., 1986) and peripheral system (Bruynzeel, 1984).

It is surprised to find that the response of LDH activity to the treatment is similar to that of MDH. Denervation decreased the LDH activity in all the muscles tested while clenbuterol reversed its effect. LDH activity increased in the soleus after acute denervation. This shows that the effect is fiber type dependent. Slow twitch muscles which utilize mainly the energy from oxidative metabolism increased its glycolytic activity after denervation. However, the enzyme activity decreased as the period of denervation was lenghtened. The decreased LDH activities in the muscles examined agree to the finding in three week denervated muscle (Simard et al., 1985).

Clenbuterol effect on denervated adult fast twitch muscles can only be obvious in rats with acute treatment. Perhaps the effect of clenbuterol on fast twitch muscle can last for only a short period. In young rats, only EDL did not respond to clenbuterol treatment. With denervation and clenbuterol administration soleus showed similar pattern in all the situation tested except that of acute treatment. It seems that the slow twitch fibers is the target of the clenbuterol effect. The high response of young gastrocnemius may be caused by the fact that being not fully developed the ratio of slow twitch fibers is still rather large.

The effect of another β-adrenergic agonist, isoproterenol, has been determined in skeletal muscle (Li and Jefferson, 1977). It was found that oxidative metabolism and lactate production increased in isolated rat hemicorpus after perfusion with this drug. In our experiment, however, increased oxidative metabolic activity was found only in control EDL with clenbuterol treatment. No glycolytic activity was found to be increased in the control muscles after clenbuterol treatment. The incompatibility of these results may be caused by the fact that the *in vivo* effect was reported in our experiment whereas in the *in vitro* experiment, the dose of drug encountered by the muscle is usually much higher.

As a conclusion, it seems that clenbuterol has an effect on denervation caused metabolic changes and thus the ameliorative effect of the drug on denervated muscles has a metabolic basis. Since there is no adrenergic structure at the neuromuscular junction, the function of clenbuterol can only be achieved through blood transportation or modulation of the cholinergic activity at the junction (Beani *et al.*, 1985). Therefore, the specific effects of clenbuterol on denervated muscle may be caused by the fact that the effect through cholinergic modulation is no longer available in denervated muscles. The metabolic pattern in clenbuterol treated denervated muscle may be restored at least partially to the normal one. However, the amino acid aminotransferases activity has not been assayed in our experiment and it is not clear if amino acids are still the

major energy source after clenbuterol treatment. But as the LDH and MDH activities has been restored nearly to their control level, the importance of amino acids as the energy source must be lowered. It still cannot be concluded whether the effect of clenbuterol on liver weight is a direct effect of the drug or a consequence of the metabolic changes in the skeletal muscles.

A clue to this problem can be obtained indirectly by assaying the proteolytic activities in clenbuterol treated muscles. If clenbuterol declines the increased proteinase activities induced by denervation, the supply of amino acids as the energy source will be limited. Also, as denervation atrophy is a result of increased protein degradation in skeletal muscles (Goldberg, 1969b), the ameliorative effect may be a consequence of restoring the proteinase activities. Although the restoration effect of clenbuterol on denervated muscles was reported to be contributed mainly by a large increase in protein synthesis, a significant portion of this effect is still contributed by reduced degradation (Maltin et al., 1987b). Thus it is actually possible that clenbuterol lowered the proteinase activities in denervated muscles.

Acid proteinase and cathepsin B were studied in our experiments. Acid phosphatase, being representative of lysosomal activities, was also studied. It was reported that clenbuterol promoted the growth of normal muscle in young rats (Maltin et al., 1986a). The effect of

clenbuterol in control muscle of adult and young rats was investigated. In the muscles examined, a significant effect was found only in adult soleus with long term clenbuterol treatment. However, this provides the biochemical basis for the finding that the drug had an inhibitory effect on protein degradation in normal muscle (Reeds et al., 1986).

For the proteinases studied, all increased their activities in denervated muscles. This agrees to the results in various reports (McLaughlin et al., 1974; Maskrey et al., 1977). Clenbuterol treatment had an effect on the proteinase activities in denervated muscles. Clenbuterol restored the denervation induced higher proteinase activities in only EDL and young gastrocnemius only. However, it has been reported that the effect of clenbuterol in slow twitch oxidative (type 1) fibers was particularly marked (Maltin et al., 1986b). This seems to be contradictory to our findings.

The discrepancy between these results may give some information about the mechanism of the effect of clenbuterol on denervated muscles. Maltin et al. (1987b) has shown that clenbuterol induces growth in innervated and denervated rat soleus muscle via different mechanisms. They suggested that clenbuterol exerts its effect by elevating the protein synthetic activity in denervated soleus but decreases the protein degradation in innervated muscle. Our result may suggest that clenbuterol fulfills its function in different type of denervated muscles through diverse mechanisms. In the slow twitch muscle, a higher protein synthesis rate

corresponds to amelioration of the denervation induced atrophy while in the fast twitch muscle, a decreased level of protein degradation was the explanation.

Since ß-adrenergic agonist exerts its function through the ß-adrenergic receptors, the differential effect of clenbuterol on different denervated muscles may be caused by a difference of the receptor in the muscles. It has been reported that ß-adrenergic receptor density and isoproterenol-stimulated adenylate cyclase activity were considerably greater in the slow-twitch oxidative soleus muscle than in the mixed fiber type gastrocnemius (Williams et al., 1984). So, it is not surprise to find that soleus has a higher response to clenbuterol. On the other hand, It has been reported that the maximum number of ß-adrenergic binding sites increased in young gastrocnemius after 25 days of denervation (Banerjee et al., 1977). This also agrees well with our results in which young gastrocnemius was found to be significant in restoring the denervation induced change after clenbuterol treatment. However, the question to ask is whether denervation has different effects on the ß-adrenergic receptors in different types of muscles.

It has been reported that the slow-twitch muscle adenylate cyclase activities which was usually activated by ß-agonist were completely and permanently lost on denervation. In fast twitch muscle, however, the enzyme activities recovered almost completely after an initial reduction (Festoff et al., 1977). This illustrates that

denervated fast muscles will respond to ß-therapy differently from the denervated slow muscles. The higher response of slow twitch muscles may be a consequence that addition of ß-agonist compensates the lower adenylate cyclase activity after denervation to maintain the normal function of ß-stimulation on this muscle. As it seems that denervation functions on fast twitch muscle not through the effect on ß-stimulation, it is reasonable that ß-therapy has no effect on denervation induced atrophy in fast twitch muscles.

As the membranous organelles in denervated muscles were found to be enlarged under the electronmicroscope (Pellegrino and Franzini, 1963; Stonnington and Engel, 1973), it was suspected that if lipid peroxidation was involved in the denervation process. It was because that increased lipid peroxidation will cause damage to the membranous structures.

In the reports concerned so far, level of lipid peroxide in denervated muscle has not been studied. For this reason, amount of lipid peroxides in denervated and clenbuterol treated muscle were determined in our experiment. Malonaldehyde (MAD) or generally referred to as the thiobarbituric acid-reactive product was used as an index of the level of lipid peroxides in muscle homogenates. In all the muscles studied, no change in MAD level was found. This may imply that there was no accumulation of lipid peroxides in the muscles examined.

Another way to investigate the involvement of lipid

peroxidation in denervation induced damage is to determine the activities of the enzymes involved in scavenging the free radicals. Change in MnSOD activity was not found in both the denervated and clenbuterol treated muscles. CuZnSOD activity as well as total SOD activity increased only in young gastrocnemius and adult EDL after denervation. Clenbuterol restored the denervation caused change to its control level.

Since MnSOD is located in the matrix of mitochondria, its concentration is usually used as an indicator of the number of mitochondria in muscle (Mizuno, 1984). MnSOD concentration also reflects mitochondrial free radical production and this is in turn a function of mitochondrial metabolic activity (Asayama et al., 1986). The stable MnSOD concentration in the muscles examined may imply an unchanged mitochondrial metabolic activity. However, this is contrary to the change of MDH activity which represents the mitochondrial activity in our experiment. Also, the result of MnSOD we obtained differs from that of Asayama et al. (1986). They found a decreased MnSOD activity in denervated muscle.

Increased CuZnSOD activity can be found only in the denervated fast twitch muscles. Clenbuterol reversed this effect. This pattern of clenbuterol influence is similar to that of the proteinases assayed. So, a muscle type specific effect was also observed. The increased CuZnSOD activity in denervated muscles suggest an increased turnover of active

oxygen species. As CuZnSOD is a cytosolic enzyme, the higher activity implies that the oxygen species have a cytosolic or extracellular origin. Our result is, however, opposite to that of Asayama et al. (1986). They found a mild decreased CuZnSOD activity in slow twitch muscle. The different result may be an outcome of the different methods employed. They measured the immunoreactive activities while we measure the ability of the enzyme to inhibit the autoxidation of epinephrine.

The similar pattern of proteinases and CuZnSOD activity in muscles after clenbuterol treatment shows CuZnSOD may be related to the lysosomal activities in skeletal muscle. However, the significance of this relation is not clear. Perhaps more free radicals are produced in the denervated muscles and then induced a higher CuZnSOD activity. The free radicals raised by denervation may function on proteins and thus make them more susceptible to the degradation of proteinase. It has been shown that oxidized proteins were more susceptible to proteolytic activities (Rivett and Levine, 1987).

Contrast to the result of SOD, catalase activity increased in all denervated muscles. Clenbuterol treatment usually lowers this activity in denervated muscle. If catalase can actually be used as an enzymatic indicator of the degree of muscle wasting as suggested by Stauber et al. (1977), then our result can be explained by that denervation causes muscle degeneration and thus increases catalase activity but clenbuterol treatment reverses the wasting

process and thus lowers the catalase activity. However, the diverse effect of clenbuterol on different type of muscles cannot be found. This may reflect that catalase activity is much more sensitive to muscle atrophy than the gross change. Thus although SOD and catalase are closely related their activity is not necessary to respond to operation and drug treatment similarly. The reason for the increase of catalase activity in denervated muscle is not clear. It may be an actual increase of production of the enzyme in denervated muscles or a consequence of invasion of other cells which contains catalase.

For the time effect investigated, decreased clenbuterol response was found only in a few enzyme activities in the two week treated rats. So, it is rather unlikely that desensitization occurred during clenbuterol treatment. However, a treatment of longer time may have this effect. This is worth studying in the future.

As a conclusion, by examining the weight it was found that EDL did not respond to clenbuterol treatment. For energy metabolizing enzymes, the same pattern was shown in both LDH and MDH activities, with the differences in the response of different muscles types. All muscles showed the same response to denervation and clenbuterol treatment in MDH activity. For LDH activity, fast twitch muscle did not respond to clenbuterol treatment. As LDH determines the glycolytic activity, lower LDH activity in fast twitch muscle made it short of energy supply. The insensitivity of

fast twitch muscle to clenbuterol in restoring its LDH activity may be explained that EDL atrophy goes on even after the institution of clenbuterol treatment.

For proteinases and SOD, all respond to denervation by increasing their activities but only the fast twitch muscles showed response to clenbuterol treatment. Thus, it was suggested that clenbuterol effected its functions in different muscle types through diverse mechanisms. That in the slow twitch muscles may depend on the ß-adrenergic system. Pattern of catalase activity with the treatment was the same in all the muscles. As its pattern did not agree to that of the other free radical scavenging enzymes, its function was not clear.

It has been shown that another ß-adrenergic agonist, isoproterenol, also decreased protein breakdown in skeletal muscle (Li and Jefferson, 1977). Also, drugs of ß-agonist group such as fenoterol and cimeterol have also been reported to have muscle specific anabolic action (Maltin et al., 1987b). Thus it is valuable to determine the effect of these ß-agonists on skeletal muscles. This may be helpful to clarify the effect ß-agonist agonists on muscle growth.

On the other hand, Maltin et al. (1987a) has reported that ß-antagonist, propranolol, did not lower the ability of clenbuterol to stimulate protein accretion but reduced the increase in muscle fiber size. They found that ß-agonist did not impair the ability of clenbuterol to stimulate muscle growth but significantly reduced the effects of ß-

agonist on body fat and, perhaps more importantly on total energy expenditure. They suggested that although some of the effects of clenbuterol are truly ß-mediated, the particular effects of this drug on muscle protein accretion bear little if any relationship to its action as a ß-agonist. Therefore, by investigating the effect of other ß-agonists and the effect of clenbuterol together with ß-antagonists will give a clearer picture of the function of clenbuterol on muscle growth. The function of ß-adrenergic receptors in skeletal muscle can thus also be clarified.

The effects of clenbuterol investigated so far are mainly that on the enzyme activities. However, since clenbuterol shows effect on growth of animals, it is probably that hormones involved in growth may also participate in the clenbuterol induced effect. It has been reported that growth hormone increased protein synthesis in muscle without changing protein degradation rates (Goldberg, 1969a). This effect is quite similar to that of clenbuterol on denervated muscle as reported by Maltin (1987b). On the other hand, insulin has effects on muscle growth and maintenance and it has been that functions of insulin on skeletal muscle decrease after denervation (Burant et al., 1984; Smith and Lawrence, 1984; Smith and Lawrence, 1985; Donalson and Harrison, 1986; Smith et al., 1988). It is possible that denervation lowers the hormone response of muscle and causes the atrophy while clenbuterol increase the hormone concentration or the hormone response of denervated

muscles to ameliorate the denervation caused effect. Emery et al. (1984) has reported that clenbuterol did not affect plasma insulin, growth hormone, or triiodothyronine levels in rats. So, it is also valuable to test the effect of clenbuterol on the hormone response of skeletal muscles.

CHAPTER 5 GENERAL DISCUSSION

In this study, the muscle enzyme activities in two animal models of muscle atrophy were investigated.

Atrophy of denervated muscle was assessed by the decreased weight while that of the muscle in ethanol treated rat was confirmed by the increase in alkaline phosphatase activity in the muscles.

By examining the energy metabolizing enzymes, after ethanol treatment, gastrocnemius was found to be dependent more on oxidative metabolism. EDL did not show a shift in metabolic activities. Soleus, however, shifted its metabolic pattern from that of oxidative to anaerobic glycolysis. In denervated muscles, all decreased the activities of enzymes of glycolysis and citric acid cycle. The different energy metabolizing enzyme patterns of these two forms of muscle atrophy indicate rather different mechanisms in these atrophying muscles. When clenbuterol was administrated to denervated animals, it resumed the denervation induced decrease in MDH activity in all muscles and LDH in slow twitch muscles. As slow twitch muscle obtains its energy mainly from oxidative metabolism, the fact that glycolytic enzyme activity but not that of oxidative metabolism was restored in clenbuterol treated muscles shows that the metabolic change in denervated muscles is a consequence of other changes, such as an

increased proteolytic activities. Both ethanol and clenbuterol treatment showed a muscle type specific effect.

For the proteinases examined, their activity increased in all muscles undergoing atrophy although in ethanol treated rat the effect was not significant. The result in ethanol treated rat suggests that probably proteinases other than the acidic proteinases which were studied in our experiment were involved in this form of muscle degeneration. Clenbuterol has its effect on proteinase activities only in the normal soleus and the denervated fast twitch muscles. This result agrees to the finding of Maltin et al. (1987b) that clenbuterol decreases protein degradation in normal soleus but not in denervated soleus. It also suggests that clenbuterol exerts its action in different types of denervated muscles through diverse mechanism. It decreases proteinase activities in denervated fast twitch muscle but increase the protein synthesis in denervated slow twitch muscle.

As calpain activity has been reported to be increased in denervated muscle (Elce et al., 1983) and the increased activity was mainly found in fast twitch muscle (Hussain et al., 1987), it is interesting to investigate whether clenbuterol has a fast twitch muscle specific effect on its calpain activity. The results of the ethanol treated rat revealed that the atrophy in these muscles is still at a early stage and the extent is still not quite serious. This is because calpain is believed to function at the early stage of muscle atrophy for its neutral optimum pH and its

ability to degrade Z-line (Otsuka et al., 1988). Acidic proteinase are believed to function only when the atrophy has further developed to a more advanced because they can degrade only the disassembled myofibrils and function in the non-physiological acidic environment (Schwartz and Bird, 1977; Katunuma and Noda, 1982; Matsukura et al., 1984).

Muscle type specific effects were also observed in the free radical scavenging enzyme activities. For alcoholic myopathy, only fast twitch muscles showed altered activity in response to the treatment. However, it was soleus which underwent atrophy. Therefore, it seems that the lacking in adaptation to the increased free radical production renders the slow twitch muscle susceptible to the free radical damage.

Catalase and SOD activities showed different change after denervation and clenbuterol treatment. CuZnSOD activity did not change in slow twitch muscle. This activity increased in denervated slow twitch muscle and clenbuterol reversed this change. The different change of CuZnSOD activity in slow and fast twitch muscles also suggests different mechanism of neural control. The elevated CuZnSOD activity in denervated muscles suggests a higher turnover of active oxygen species and the situation in clenbuterol treated muscle suggests the turnover rate has been restored to the normal level. However, catalase activity shows a peculiar sole pattern in the clenbuterol treated denervated muscles. It is not certain at the moment

what the altered catalase activity implies.

By examining the result of clenbuterol treated muscles, it is found that clenbuterol has its effect on both normal and denervated slow twitch muscles but only in denervated fast twitch muscles. It is proposed that in slow twitch muscle part of the normal function is maintained through the β-adrenergic system. While in denervated muscles, without the control of the cholinergic system, the effect of the β-adrenergic system is predominates.

Besides protein degradation and lipid peroxidation, there is another system which may be related to the damage seen in muscle atrophy. This system involves prostaglandins and arachidonic acid. It has been shown that they are involved in regulation of protein turnover in skeletal muscles (Rodemann and Goldberg, 1982; Barnett and Ellis, 1987; Reeds et al., 1987). It is quite possible that the treatment disturbs the regulation of this system and thus change the balance of protein turnover, thus causing the damage. Therefore, it would be valuable to detect the activities of components of this system in the myopathy models.

The normal function of skeletal muscle is also maintained by hormones such as insulin. It has also been reported that response to insulin decreased in denervated muscles (Burant et al., 1984; Smith and Lawrence, 1985; Smith et al., 1988). It is quite possible that the lacking in sensitivity to this hormone in the diseased muscles triggers the atrophy process. It would therefore be

informative to determine the effect of this and other hormones in the normal and atrophying muscles.

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